SEDIMENT QUALITY ASSESSMENT

A PRACTICAL GUIDE

SECOND EDITION

EDITORS: STUART SIMPSON AND GRAEME BATLEY

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Front cover: Low tide on Cid Island in the Whitsunday group in Queensland, showing relatively pristine exposed coastal sediments and some of the associated organisms. Credit: Rob Jung, CSIRO.

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Preface

The last decade has seen an exponential growth in our understanding of the forms, fate and effects of contaminants in sediments. In Australia, as in many parts of the world, sediment quality guidelines are now well established in regulatory frameworks. However, detailed guidance on how to interpret and apply the guidelines is generally limited. It is recognised that additional research is needed to resolve several uncertainties in the science underpinning sediment quality guidelines. In Australia and New Zealand, the approach has been to introduce a tiered assessment framework so that exceedance of the sediment quality guideline value leads to additional studies to confirm or deny the possibility of biological impacts. This approach is outlined in the *Australian and New Zealand guidelines for fresh and marine water quality*, published in 2000.

In 2010, the sediment quality guidelines for Australia and New Zealand were revised (by the editors and an author of the present volume). The revision introduces the use of multiple lines of evidence in a weight-of-evidence approach to the assessment. This is consistent with similar developments internationally. The approach extends the current decision framework so that it provides a means, where necessary, of combining lines of evidence. For example, bioaccumulation and ecological assessments can now be combined with the traditional chemistry and laboratory toxicity lines of evidence, and there is now a mechanism for bringing in additional information on chemical exposure and bioavailability which can improve assessments of causality.

Thus, now, investigations should ideally combine assessments of:

- sediment chemistry (such as exceedances of sediment quality guidelines), including contaminant bioavailability tests (for example, pore-water measurements, acid volatile sulfide tests, passive sampling methods and approaches that mimic biotic responses to hydrophobic organic contaminants);
- toxicity testing (for example, of multiple species, varying exposure pathways, and acute and chronic endpoints such as survival, growth, reproduction or avoidance, and biomarkers of effects);
- bioaccumulation or biomagnification; and
- benthic community structure and function.

Toxicity identification evaluation (TIE) and other assessments of causality may also be of value. The combination and interaction between lines of evidence should be considered in applying these in a weight-of-evidence framework (for instance, particle size affects contaminant bioavailability, and bioavailability test results will affect the interpretation of toxicity and bioaccumulation data). Weight-of-evidence assessments often ultimately rely on best professional judgment, but the use of tabular decision matrices is the best approach for achieving transparency and comprehension by personnel outside the field of ecological risk assessment.

Environmental practitioners are seeking guidance on how to incorporate the latest science in their assessment of contaminated sediments, while relating their investigations to the recommended guideline frameworks, and proposed new or revised guideline values for sediment quality, at a time when the science is still being developed. This handbook therefore attempts to summarise the advances and provide information to guide future sediment quality assessment investigations.

The book both reviews the existing literature and recommends best ways to apply these findings, while describing approaches for measuring the various lines of evidence. As new lines of evidence are continuing to be developed, future sediment quality assessments may also incorporate those. A general approach is proposed, recognising that assessments frequently need to be custom-designed and lines of evidence chosen to suit the site-specific circumstances (such as site dynamics, sediment stability, groundwater flows, and fluctuating overlying water conditions).

The focus on sediment quality assessment, at least in Australia, has largely been in estuarine and coastal marine environments, but the principles are equally applicable to freshwater systems, and guidance is therefore also provided in this book for freshwater toxicity testing and ecological assessment procedures for freshwater environments.

Stuart L. Simpson
Graeme E. Batley

About the editors

Stuart Simpson is a Senior Principal Research Scientist and leader of the Aquatic Contaminants Group in CSIRO Land and Water. He has more than 20 years' research experience, covering water and sediment quality assessment in freshwater and marine environments. His research interests include establishing relationships between contaminant forms, bioavailability and exposure and observed biological/ecological effects, and the development and application of sediment quality guidelines and advanced assessment tools. Aspects of that research led to the award, with Graeme Batley and Jenny Stauber, of the Land and Water Australia Eureka Prize for Water Research in 2006. Stuart is author of some 200 research publications.

Graeme Batley is a Chief Research Scientist with CSIRO Land and Water and past Director of the Centre for Environmental Contaminants Research based in Sydney, Australia. He is one of Australia's leading researchers of trace contaminants in aquatic systems, actively researching this area for over 40 years. He was a lead author of the water and sediment quality guidelines for Australia and New Zealand in 2000 and of the *Australian guidelines for water quality monitoring and reporting*, and has recently led the updating of toxicant guidelines for both waters and sediments. Graeme is author of over 400 scientific publications.

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disturbance ecology, ecotoxicology, biometry, and understanding the ecological ramifications of sea-level rise on coastal environments. Anthony is an Adjunct Associate Professor at the University of New South Wales and University of Canberra, and is an Associate of the Canadian Rivers Institute, University of New Brunswick.

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Donald Baird is a Senior Research Scientist in the Water Science and Technology Directorate of Environment Canada. He is also a Visiting Research Professor in the Biology Department at the University of New Brunswick and a Science Director at the Canadian Rivers Institute. His research team is focused on the measurement of aquatic biodiversity, diagnostic biomonitoring, ecological flow needs for rivers, and ecological risk assessment. Donald is also pioneering the application of ecogenomic approaches in ecosystem assessment, focusing on boreal floodplain wetlands of the Peace–Athabasca Delta currently threatened by expanding resource development in the region.

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Other contributors

While this handbook represents a major re-write and extension of the 2005 *Handbook for Sediment Quality Assessment*, we acknowledge the contributions of the following scientists to that older document:

Jenny L. Stauber, Catherine K. King, John C. Chapman, Ross V. Hyne, Sharyn A. Gale, and Anthony C. Roach.

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1

Introduction

Graeme E. Batley and Stuart L. Simpson

1.1 Background

Sediments are the ultimate repository of most of the contaminants that enter Australia's waterways, and therefore it is appropriate that regulatory attention addresses the ecological risks that sediment contaminants might pose. There is increasing public awareness of, and concern for, the health of our waterways, and an expectation that water quality will be improved, but any improvement in water quality must address sediments as an important component of aquatic ecosystems and a source of contaminants to the overlying waters and to the ecosystem through the benthic food chain.

The sediments of many of the urban river systems, estuaries and near-shore coastal waters worldwide have high contaminant loads, derived largely from past industrial discharges and urban drainage. In many instances, there are elevated concentrations of nutrients, metals and metalloids and organic contaminants, especially polycyclic aromatic hydrocarbons (PAHs). Where regulations are adequate and met, the licensing of discharges has effectively controlled contaminant concentrations reaching surface waters from point sources; however, their concentrations in sediments often remain a concern. In many developing countries, regulations are weak and often not enforced. In highly urbanised areas, urban drainage, including road runoff, continues to represent a major source of contaminants that ultimately accumulate in sediments. Major point sources, such as partially treated sewage and discharges from mining and various light industries, contribute significantly. Rainfall events can result in leaching of contaminated land sites, with contaminants reaching surface waters and groundwater, both of which can contribute ongoing contamination to sediments.

Typically, as part of the management of contaminated sites, it is required that the risk of harm from any potential contaminants be assessed before the sites undergo any major disturbance through redevelopment or remediation orders placed on them. This involves an assessment of the potential toxicity, persistence, bioaccumulation, and fate and transport of the contaminants. Management and/or remediation of contaminated land and sediments is costly and needs to be based on sound science.

A range of sediment quality guideline values (SQGVs) for contaminants have been proposed internationally (Buchman, 2008) and they form the basis of assessments of the risk that sediment contaminants might pose to the environment. The sediment quality guidelines within the main water quality guidelines for Australia and New Zealand

(ANZECC/ARMCANZ, 2000a) have recently been revised (Batley and Simpson, 2008; Simpson *et al.*, 2013). Besides minor changes in some SQGVs, the revision outlines a scheme for the integration of multiple lines of evidence in a weight-of-evidence framework to be used in decision-making in cases where the results from chemistry and toxicity testing are equivocal. This reflects the latest in international thinking in relation to sediment quality assessment.

The original edition of this sediment quality assessment handbook (Simpson *et al.*, 2005) was largely the output from a project to develop protocols for assessing the risks posed by metal-contaminated sediments. The project, funded by the NSW Environmental Trust, was undertaken jointly with researchers from University of Canberra and the NSW Office of Environment and Heritage. That study developed sensitive new sediment toxicity tests for estuarine–marine environments, examined metal uptake pathways for sediment-dwelling organisms, and characterised metal effects on sediment communities. In this new edition of the handbook, the information gained in those studies and related research conducted by the team has been integrated with the latest international research, to provide a more sound and practical basis for sediment quality assessment.

Since 2005, there have been several advances in methods for sediment quality assessment. A range of new whole-sediment toxicity tests have been developed covering both acute and chronic exposures. These tests have led to an improved understanding of controls on contaminant bioavailability and uptake pathways that can be used to refine the SQGVs. Biomarkers are being increasingly used to provide evidence of sub-lethal effects, while advances in ecogenomics are beginning to dramatically improve assessments of biodiversity in sediments. The improvements in these lines of evidence are coupled with advances in the application of weight-of-evidence assessments.

1.2 Sediment monitoring and assessment

There are several reasons why a sediment quality assessment might be undertaken. These might include:

- measurement of baseline concentrations at a pristine location;
- mapping the contaminant distribution in sediments in a waterbody to assess the distribution of historical inputs;
- determining the impact of known inputs (examples include stormwater runoff, industrial discharges, mining discharges, sewage and wastewater treatment plant inputs, shipping activities);
- assessing sediments requiring remediation (dredging, capping); and
- assessing the impacts of dumped sediments (from dredging activities).

These fall into three distinct categories: (i) descriptive studies; (ii) studies that measure change; and (iii) studies that improve system understanding (cause and effect). The assessment approach may be slightly different for each of these depending on whether the primary focus is on contaminant distribution, ecosystem health, or the potential for toxic impacts.

The first step in any assessment process (Fig. 1.1) is therefore the setting of the objectives (ANZECC/ARMCANZ, 2000b; USEPA, 2002a). As part of this process, the issue to be investigated (such as those in the list above) is determined, together with information requirements. Existing information is collated to help define a system understanding. This is then displayed in a conceptual process model which encapsulates all of the likely receptors and processes associated with the movement of contaminants and other stressors associated with

the sediments. Examples of conceptual models for sediments are shown in Fig. 1.2 for biological receptors and their potential contaminant exposure routes, and in Fig. 1.3 for major contaminant processes influencing partitioning between water and sediment.

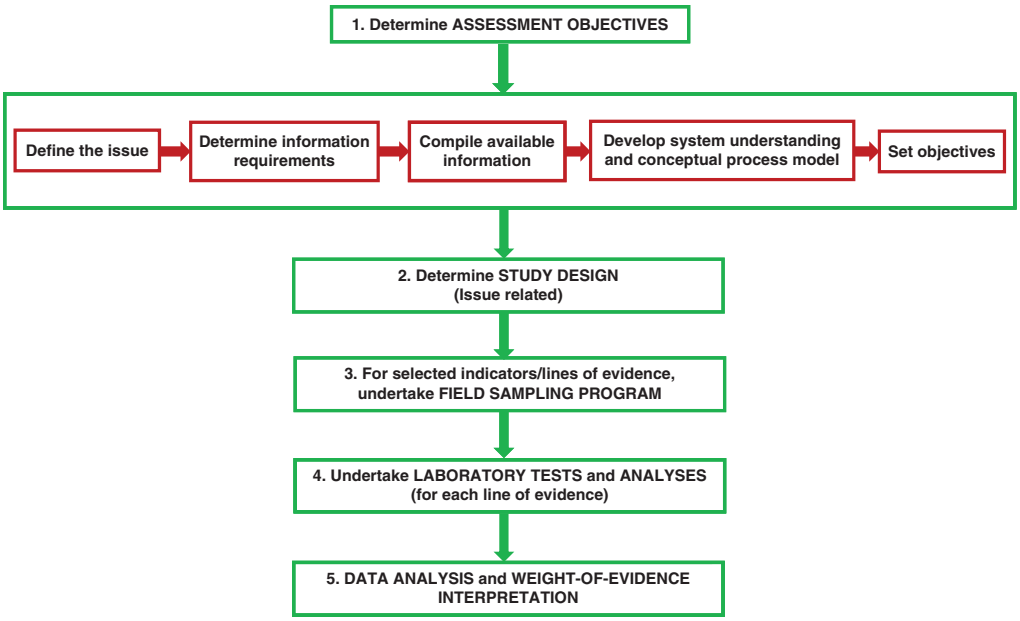

Figure 1.1. Monitoring and assessment framework for sediment quality investigations.

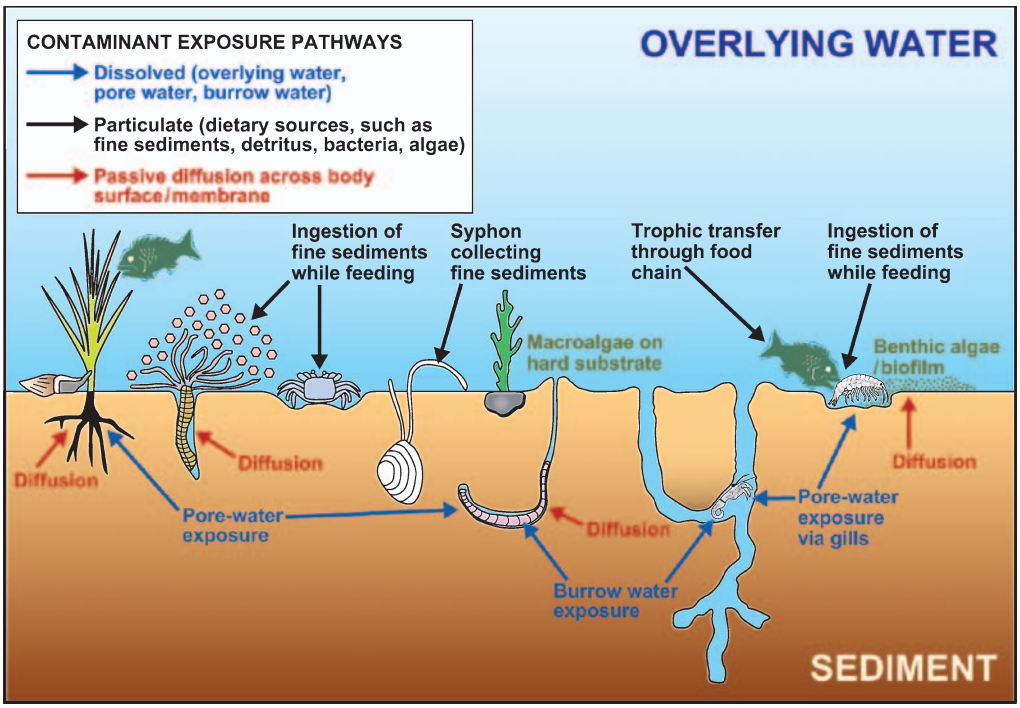

Figure 1.2. Conceptual model of organisms, receptors and potential exposure routes in sediments.

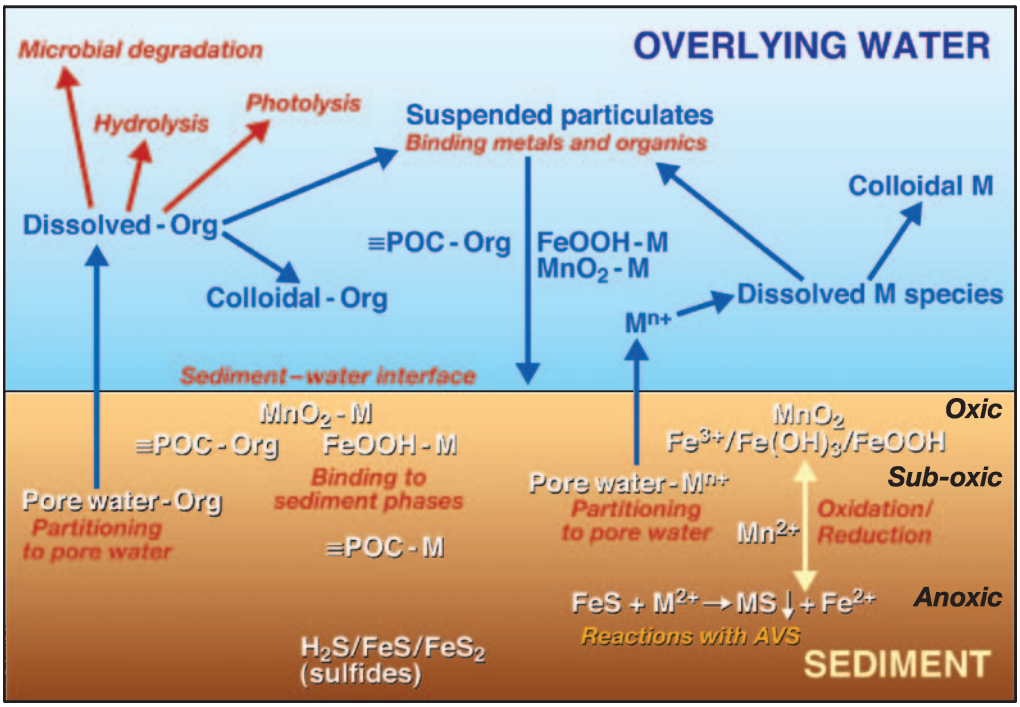

Figure 1.3. Conceptual model of major contaminant processes in sediments (where M indicates 'metal', POC is particulate organic carbon, and Org refers to organic compounds, so POC – Org is organics associated with POC).

The next step is to determine the study design, defining the indicators or measurements and tests to be made (the lines of evidence to be investigated) and developing the field sampling and analysis plan. After executing the field sampling and laboratory analyses, the final step is data analysis and interpretation on the basis of the weight of evidence (USEPA, 2002b,c). As a consequence of the data analysis, a need for lines of evidence additional to those originally chosen might be identified, leading possibly to a revised conceptual model, or at least a revised study design.

1.3 Sediment quality guideline values (SQGVs)

A key component of the assessment of sediment chemistry is the comparison of measured contaminant concentrations against SQGVs. Guideline values for Australia and New Zealand were released in 2000 and represented the latest in international thinking at that time (ANZECC/ARMCANZ, 2000a), but they have recently undergone revision (Simpson *et al.*, 2013). Empirical SQGVs had already been adopted in Canada, Hong Kong and several states of the USA, and were also being considered in Europe (Babut *et al.*, 2005; Buchman, 2008). In Australia in 2000, unlike elsewhere, the SQGVs were to be used as part of a tiered assessment framework (Fig. 1.4) in keeping with the risk-based approach introduced in ANZECC/ARMCANZ (2000a). As indicated later, SQGVs are considered during the evaluation of the 'chemistry' line of evidence (Section 1.4) but were derived through consideration of matching chemistry and effects data.

There have been two approaches to the derivation of SQGVs: (i) empirically-based, and (ii) mechanistic approaches that are based on equilibrium partitioning (EqP) theory

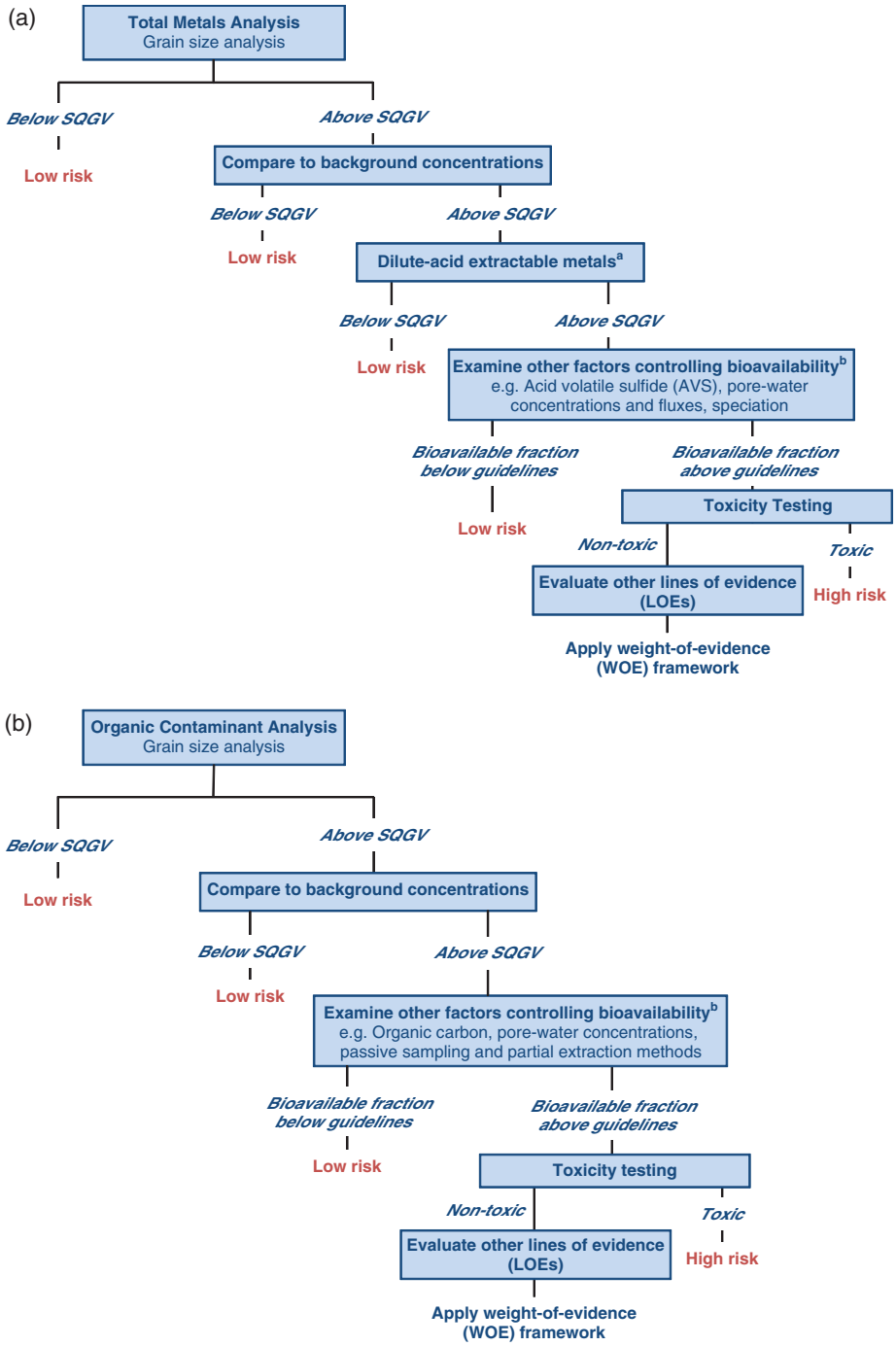

Figure 1.4. The tiered framework (decision tree) for the assessment of contaminated sediments for (a) metals, and (b) organics. SQGV = sediment quality guideline value. Notes: [a] This step may not be applicable to metalloids (As, Se) and mercury (Hg). [b] See specific methods on how bioavailability test results are used (Chapter 3 Section 3.6). Other lines of evidence may be considered using readily available tools for assessing toxicity, bioaccumulation, ecology impacts, or other lines of evidence such as biomarkers (see Section 1.4, Fig. 1.5).

(Batley *et al.*, 2005). The various versions of both approaches frequently converge in the prediction of effects on benthic organisms. In short, the science is able to define reasonably well the concentration ranges below which no effects are observed and above which effects are almost always observed. However, in the intermediate 'transition zone' the predictions become poor, in some cases varying by as much as an order of magnitude.

Australia and New Zealand adopted empirical SQGVs derived from a ranking of toxicity data and other effects data, from field studies using a large North American database. While both lower and upper guidelines were provided (termed 'SQGV' and 'SQGV-high', respectively), equivalent to the ERL ('effects range low') and ERM ('effects range median') introduced by Long *et al.* (1995), regulation was based on the lower guideline. By definition, there was a low probability of effects below the lower guideline value and a high probability above the upper guideline value. The lower value (the SQGV) is used as a screening value; if exceeded, it is a trigger for further investigation.

Unlike the guideline values for *water* quality, the Australian and New Zealand SQGVs are not based on cause–effect relationships. This has sometimes caused confusion and misinterpretation of the ecotoxicological significance of the sediment chemistry data.

The empirical approach uses the 10th percentile and median of the ranked effects data to derive the two guideline values. Sediments typically contain co-occurring contaminants (such as metals and organics), but in ranking the data any observed toxicity is equally attributed to all components of the mixture. As a consequence, the derived SQGVs can be quite conservative. For example, consider a sample containing zinc at low concentrations and PAHs at high concentrations; toxicity of this sample would be ascribed equally to the zinc (which is not necessarily causing any effects) and the PAHs; in this case, the derived SQGV for zinc would be over-protective.

A measured value that exceeds the SQGVs does not necessarily mean that adverse biological effects will occur in the sediments but instead that further investigations should be undertaken to confirm the likely effects, following the site-specific tiered assessment frameworks shown in Fig. 1.4. Such investigations usually involve a consideration of the bioavailable concentration and then, if this still exceeds the SQGV, further lines of investigation are pursued (by examining additional lines of evidence). In most instances, the next line of evidence in the framework involves toxicity testing. Other lines of evidence might include bioaccumulation and sediment ecology (Simpson *et al.*, 2013).

The latest Australian and New Zealand SQGV and SQGV-high values are summarised in Appendix A. A good summary of international SQGVs is provided by the USA National Oceanic and Atmospheric Administration (NOAA) in its screening quick reference tables (SQuiRT) (Buchman, 2008).

1.3.1 Advances in the derivation of SQGVs

As already discussed, the several limitations in the currently accepted empirical SQGVs are to some extent overcome by restricting SQGVs to use as screening values only. For copper- and nickel-spiked sediments there have been trials applying species sensitivity distributions to whole sediment toxicity data (Simpson *et al.*, 2011; Campana *et al.*, 2013; Vangheluwe *et al.*, 2013), but it was found that toxicity was strongly influenced by sediment properties (discussed in Chapter 3 Section 3.6). For Cd, Cu, Ni, Pb and Zn, the influence of acid volatile sulfide (and, potentially, of organic carbon) on the bioavailability of these metals forms the basis of a mechanistic-based approach to deriving SQGVs (Chapter 3 Section 3.6.1). For major non-ionic organic chemicals (such as hydrophobic organic contaminants, HOCs), equilibrium partitioning models based on partitioning to organic carbon provide an alternative form of guideline that is also anchored to effects data (discussed in Chapter 3 Section 3.6.3).

For all SQGVs, the success of the approach depends on the number and quality of the available tests. The European Commission's Water Framework Directive (European Commission, 2011) recommends the use of long-term whole-sediment laboratory toxicity tests with sediment organisms and spiked field sediments. Assessment factors are applied to the tests as follows: for one long-term test (EC10 or NOEC), divide by a factor of 100; for two long-term tests with species representing different living and feeding conditions, divide by 50, and for three such tests, divide by 10. A factor of 1000 is used for short-term tests.

Again, as noted for water-quality guideline values, the use of assessment factors is not the preferred approach. Instead, the application of species sensitivity distributions to datasets containing at least eight species from four taxonomic groups is recommended. In the case of sediments, however, it is recognised that these minimum data requirements will rarely be met.

1.4 Using multiple lines of evidence

The traditional consideration of only contaminant chemistry and ecotoxicology is not always sufficient to determine whether sediment contaminants are affecting ecosystem health. It is therefore appropriate that the decision trees in Fig. 1.4 include a consideration of other lines of evidence, as shown in Fig. 1.5.

Situations that would dictate this might include:

- the presence of major contaminants for which there are no SQGVs;
- the presence of an unknown mixture of contaminants at a site;
- confounding results being obtained from chemical assessment and toxicity testing (that is, exceeded SQGVs are not supported by toxicity tests; or toxicity is seen when no SQGVs have been exceeded);
- a requirement from a regulatory agency for a full ecological risk assessment of impacts on sediments from either historical, existing or proposed activities that could have impacts on sediment ecosystem health;
- an apparently degraded ecological environment that requires more detailed evaluation; or
- the site being sufficiently large and the remediation options so expensive that it is better to target treatment only to those sediments delineated as posing the greatest risks to ecosystem health.

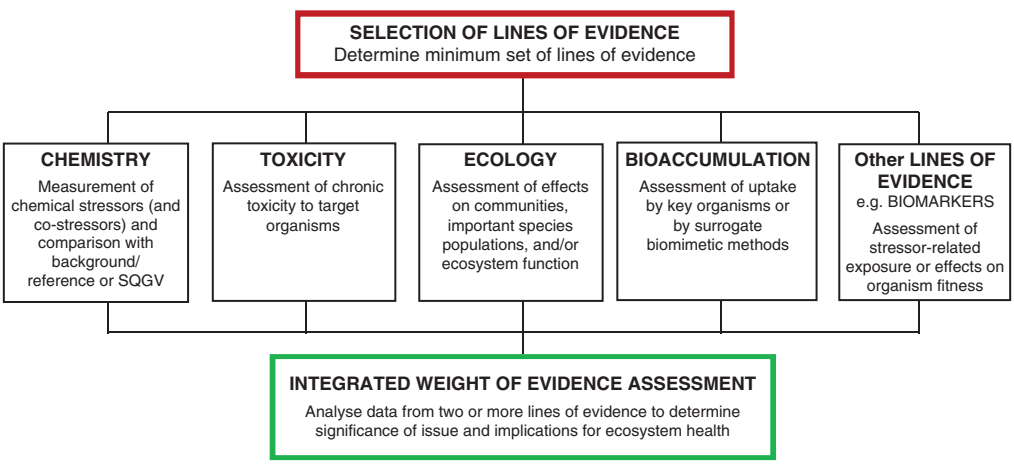

Figure 1.5. Lines of evidence for consideration in a weight-of-evidence assessment.

Lines of evidence based on chemistry and ecotoxicology are typically supplemented with measures of bioaccumulation and benthic ecology, which are important indicators of sediment quality (Batley *et al*., 2002, 2005; Simpson *et al*., 2005; Wenning *et al*., 2005). Biomarkers of sub-lethal exposure and effects can also be included along with any other lines of evidence that may usefully contribute to the assessment. Their assessment uses a weight-of-evidence framework that considers all of the lines of evidence together (Chapman *et al*., 2002; Batley *et al*., 2002; Chapman and Anderson, 2005; Simpson *et al*., 2005).

There are three basic approaches to weight-of-evidence assessments:

- qualitative methods based on best professional judgement;
- semi-quantitative approaches using rankings or scoring systems; and
- quantitative methods using probability or multivariate approaches.

Ideally, an assessment that delivers the same result regardless of who is doing the assessment is preferable to one that requires expert professional judgement. This is best achieved using semi-quantitative approaches.

A number of semi-quantitative approaches have been developed that vary only marginally. These began with the sediment quality triad (considering chemistry, ecotoxicology and ecology) (Chapman, 1990), later extending to tabular decision matrices such as that proposed by Chapman *et al*. (2002), or the framework recommended for Australia and New Zealand (Simpson *et al*., 2013) (Table 1.1) that involves scoring three levels of effect: none, moderate or high.

The use of scoring systems has been discussed by Chapman (1990, 1996), USEPA (2000) and Grapentine *et al*. (2002). Grapentine *et al*. (2002) advocated a pass (+) or fail (–) approach to each line of evidence, based on a ranking (score of 1 to 4) within each. The scheme of Bay and Weisberg (2012) uses four levels of effect applied to the traditional triad and uses indices for each line of evidence. Any of these approaches is likely to effectively rank the risk from sediment contaminants and so be of value in defining management actions.

The lines of evidence include those that form parts of the ANZECC/ARMCANZ (2000a) tiered assessment framework (Fig. 1.4), namely sediment chemistry (for example, exceedances of SQGVs), contaminant bioavailability tests (for example, pore-water measurements, acid volatile sulfide (AVS), passive samplers and biomimetic approaches for hydrophobic organic contaminants), and toxicity testing. Additional lines of evidence may include bioaccumulation/biomagnification, biomarkers, benthic community structure (such as ecological malfunction), toxicity identification evaluation (TIE) and other causality considerations. Approaches for measuring various lines of evidence are discussed in later chapters. Many new lines of evidence are continuing to be developed for sediment quality assessment purposes. There is no single multiple line-of-evidence approach for sediment quality assessments, and studies should be custom designed and lines of evidence chosen to suit the site-specific circumstances (for instance, site dynamics, sediment stability, groundwater flows, fluctuating overlying water conditions). Field-based (*in situ*) testing may be applicable for some assessments.

The more detailed quantitative approaches are described in papers by Reynoldson *et al*. (2002), Bailer *et al*. (2002) and Smith *et al*. (2002). These are better suited to very large datasets, with large numbers of reference sites. They require an expert statistician as part of the project team.

Examples of the application of the scheme shown in Table 1.1 are given in Table 1.2. A more detailed discussion of the results underpinning the rankings within each line of evidence is documented by Simpson *et al*. (2013).

Table 1.1. Weight-of-evidence scoring system adopted for Australia and New Zealand sediment quality assessments (Simpson *et al.*, 2013)

Line of evidence	Ranking		
	3	2	1
Chemistry			
Sediment	Concentration > SQGV-high	Concentration > SQGV < SQGV-high	Concentration < SQGV
Pore water[a]	Concentration > WQG HC10	WQG HC5 < Concentration < WQG HC10	Concentration < WQG HC5
Toxicity	≥50% effect *v.* control	20–50% effect *v.* control	<20% effect *v.* control
Bioaccumulation	Significantly different ($P < 0.05$) and >3 × control	Significantly different ($P < 0.05$) and ≤3 × control	Not significantly different from control
Ecology	Significant and high effects on abundance and/or diversity	Significant but moderate effects on abundance and/or diversity	No significant effects on abundance and/or diversity
Biomarkers[b]	Significantly different from control	Moderate but significant difference from control	Not significantly different from control
Other lines of evidence	An appropriate ranking of effects		
Weight-of-evidence	**Significant adverse effects**	**Possible adverse effects**	**No adverse effects**

[a] May be used as supporting evidence for exposure (bioaccumulation line of evidence) or effects (ecotoxicology line of evidence). See Chapters 5 and 4, respectively.
[b] Elutriate samples can be used where insufficient pore waters can be collected. See Chapter 2 Section 2.13.

SQGV = lower sediment quality guideline value; SQGV-high = upper sediment quality guideline value; HC10 = concentration that is hazardous to 10% of species; HC5 = concentration that is hazardous to 5% of species; WQG = water quality guideline.

It is important to stress that the majority of sediment quality assessments can produce a satisfactory conclusion by using the simpler hierarchical decision tree (Fig. 1.4) which approaches the assessment on the basis of chemistry supplemented by toxicity testing. It is recommended that during the study design there is consideration of the quality of evidence that would be obtained from different combinations of lines of evidence, and that an early judgement is made about which lines of evidence to include. In some instances it may be in the interests of those undertaking the sediment study to go directly to a full weight-of-evidence study, although that is typically more costly than a consideration of chemistry only, with or without ecotoxicological confirmation. Environmental managers will need to decide whether the advantages of a more detailed assessment justify the costs. For example, defining the area of environmental concern for a dredging activity might involve millions of dollars in additional remediation if the area to be remediated is not clearly defined.

Note also that the hierarchical approach shown in Fig. 1.4 need not necessarily begin with an assessment of chemistry, although this is most commonly done. Equally, the measurement of toxicity or ecological impairment or contaminant bioaccumulation might be the first step that leads on to other lines of evidence.

Table 1.2. Examples of semi-quantitative ranked weight-of-evidence decisions

Case	Line of evidence[a]				Weight-of-evidence score	Overall assessment
	Chemistry (metals, organics)	Toxicity	Bioaccumulation	Ecology		
1	3	3	2 or 3	3	**3**	Significant adverse effects from sediment contamination
2	3	3	2 or 3	2	**3**	Significant adverse effects from sediment contamination
3	2 or 3	3	2	2	**3**	Significant adverse effects from sediment contamination
4	2 or 3	2	1 or 2	2	**2**	Possible adverse effects from sediment contamination
5	2	2 or 3	1 or 2	2	**2**	Possible adverse effects from sediment contamination
6	2	2	1 or 2	2 or 3	**2**	Possible adverse effects from sediment contamination
7	2 or 3	2 or 3	2 or 3	1	**2**	Toxic chemical is stressing the system but resistance may have developed at community level
8	1	2 or 3	1	2 or 3	**2**	Possibility of unmeasured toxic chemicals causing effects on communities
9	1	2 or 3	1	1	**2**	Unmeasured physical or chemical causes of toxicity
10	2 or 3	1	1	2 or 3	**2**	Chemicals are not bioavailable or community change may not be due to chemicals

Table 1.2. (Continued)

Case	Line of evidence[a]				Weight-of-evidence score	Overall assessment
	Chemistry (metals, organics)	Toxicity	Bioaccumulation	Ecology		
11	1	1	1	2 or 3	**1**	Changes probably not due to measured contaminants
12	1 or 2	1	1 or 2	1	**1**	No adverse effects
13	1	1	1	1	**1**	No adverse effects
14	2 or 3	1	1	1	**1**	Contaminants unavailable

[a] Values listed in each line-of-evidence category are the highest scoring assessment in that category; e.g. under chemistry, metals may score 2, organics 3, so 3 is recorded. The greater the number of 3s recorded in a category, the greater is the weight that line-of-evidence category assumes.

When SQGVs are exceeded based on bioavailable contaminant assessment, it may be necessary to go beyond the next tier assessment of toxicity to demonstrate whether or not there are detrimental effects on ecosystem health. This is usually because of difficulties in demonstrating cause and effect relationships in toxicity testing, or because of the lack of appropriate tests that respond near the SQGVs for particular contaminants. Equally there is the issue of whether the SQGVs are reliable or artificially conservative. The extension of the tiered assessment to include lines of evidence such as contaminant bioaccumulation and benthic ecology is therefore logical, as is the assessment of the multiple lines of evidence in a weight-of-evidence framework. Other lines of evidence, such as biomarkers, may be added if useful for the specific assessment.

The weight-of-evidence framework extends and transforms the tiered approach so that it encompasses and ranks (using a tabular decision matrix) all available lines of evidence in a manner that is transparent and easy to comprehend by lay personnel.

Chapters 2–7 describe how to obtain the necessary data for each line of evidence discussed above.

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2

Sediment sampling, sample preparation and general analysis

Graeme E. Batley and Stuart L. Simpson

2.1 Sampling design for sediment quality assessments

Sampling design should be considered as a major component of the study design and broader framework applied for a sediment quality assessment program (see Chapter 1, Fig. 1.1) (ANZECC/ARMCANZ, 2000a; USEPA, 2001, 2006). First, the study type needs to be determined, and this will lead to a definition of the study scope (spatial boundaries, scale and duration), and the design of the required sampling program to achieve the data requirements. In many cases, sediment investigations are descriptive studies, simply designed to investigate the spatial and temporal distribution of contaminants, for 'state of the environment' reporting, for compliance monitoring, or to guide management actions such as dredging. In rarer instances, the objective may be to examine contaminant transport and depositional processes. In most instances, the assessment objectives are likely to be driven by regulatory requirements and evaluation of the potential impacts on ecosystem or human health.

Quality assurance (QA) needs to be addressed in any assessment exercise. This should include a consideration of the desired sampling and data quality objectives. Standard operating procedures should exist for methods that may be replicated and require audit or review to check compliance against the QA plans. The assessment of data quality objectives will usually require statistical evaluation to ensure that the right type, quantity and quality of environmental data are collected. There are several documents that provide guidance on these matters (for example, ANZECC/ARMCANZ, 2000a; USEPA, 2001, 2006).

Specific details on the design of a sampling program for an ecological assessment are provided in Chapter 7. Factors such as the depth of sediment collected, in-field processing and the degree of replication required may differ for different assessments and environments.

The design of a sampling program for sediments needs to consider:

- the number and location of sampling sites and their selection;
- spatial variability;
- sampling frequency;
- precision and accuracy;

- measurement parameters; and
- cost effectiveness.

The selection of sites and sampling program design must take into account the fact that sediments are quite heterogeneous, both chemically and physically, with contaminant distribution being very dependent on grain size. In general, contaminants that accumulate via adsorption to particles will be associated with the finest high surface area particles. Sandy and other coarse-grained sediment particles will generally have low contaminant content and will generally pose a low threat to benthic organisms.

The frequency of sampling undertaken in monitoring studies may also be dictated by the rate of sedimentation, or by changes in industries or their practices (for example, discharge conditions, 'footprint'). Sedimentation rates in waterbodies typically vary from 1–2 mm/y to 1–2 cm/y, although in tropical areas with large seasonal variability in river flows, the sediment accumulation in off-river areas can be much larger. Except in the latter cases, recent sedimentation is therefore unlikely to be seen at depths below 5 cm, so this should be noted when deciding the depth of core sections to be selected for analysis. Licensing conditions for industrial discharges are frequently reviewed, and water and sediment quality are affected both by changes to discharges and by events such as storms or spillage. For monitoring of the impacts of single industries, targeted sampling may be most appropriate.

Sampling program design may depend on the distribution of biological activity in sediments, which can be quite variable. Biota use the sediment variously as a refuge, a habitat and a food source. In the case of burrowers, for example, the acceptability of the sediment particle size for burrowing might determine their distribution, while for microorganisms the availability of organic matter or nutrient sources might be critical. Most biological activity occurs in the upper 10 cm, although some organisms can burrow to greater depths.

The depths to which sediments are sampled should be relevant to the monitoring objective. It may be appropriate, in any monitoring survey, to establish the nature of the depth profile of contaminants at the sites under consideration. For example, if the objective is to look at changes over time in the concentrations of contaminants in the top 10 cm of sediment where the sedimentation rate is less than 1 cm/y, measurements every 2–3 years or longer are likely to be more useful than annual measurements; or an alternative could be to sample only the uppermost (0–2 cm) sediment layer each year.

The size of the study area will greatly influence the type of sampling design and site selection methods that are appropriate. More detail on sampling design, including sample numbers and sampling frequency, is provided in ANZECC/ARMCANZ (2000a).

2.2 Patterns of sampling

2.2.1 Random sampling

Random sampling involves the selection of sites randomly in order to provide an unbiased assessment of the condition of the sediments within a waterbody. However, random sampling designs are quite likely to omit sites that could be important in identifying relationships between variables; for example, in estimating a benthic response or contaminant concentration in relation to a known contaminant discharge point. Also, a random selection of stations may not include a sufficient number of such key sites, because many randomly selected sites will be well away from the contaminant source. Simple random sampling is therefore not recommended.

To overcome these problems with random sampling designs, forms of systematic and stratified random designs are usually preferred; sampling each stratum of interest, for

example. Chapter 7 provides further information on these and other sampling designs. Spatial heterogeneity (both horizontal and vertical) must be taken into consideration. Sampling should involve replicate samples to determine localised heterogeneity (cluster sampling). Vertical homogeneity can be readily assessed from core samples and they are preferable to surface grab samples for this reason. Sediment deposition in a waterbody will not occur uniformly but will be dictated by complex patterns of flow. Scouring of bottom sediments is common in the channels of fast flowing rivers, for example, while deposition will occur in low-flow regions.

2.2.2 Stratified random sampling

In stratified random sampling, the system is divided into parts or strata, not necessarily of equal size, in which the variable of interest is as uniform as possible. The number of samples to be taken within in each stratum will depend on the size of the stratum and the variance within it. Strata can vary spatially or temporally. For example, the strata in a sediment may be defined on the basis of grain size (sandy versus silty). Seasonal effects on parameters are less likely in sediments, although there may be effects on the benthic ecology. If so, sampling may need to focus on the seasons in which greatest variability is expected (ANZECC/ARMCANZ, 2000a).

2.2.3 Systematic sampling

Systematic sampling involves sampling at even intervals in space or time. Spatially it usually entails sampling along transects or grids, while temporally it might involve a regular monitoring schedule, such as monthly sampling. Care must be taken to avoid biases that might occur by sampling at a fixed time or location.

Existing information is helpful in forming the sampling program design (ANZECC/ARMCANZ, 2000a). The term 'targeted sampling' is used to describe sites selected based on prior knowledge of other factors, such as contaminant sources, substrate types, water depths, tidal influences, and human activities. Targeted sampling designs can often be implemented quickly and offer much more flexibility than statistically-based (random) sampling designs. Data from targeted sampling cannot, however, be easily used to make predictions of contamination at other sites (stratified random sampling is better for this purpose).

2.2.4 Cluster sampling

Replication can be achieved through cluster sampling; that is, collection of several pseudo-replicated samples in close proximity, as discussed further in relation to ecological sampling in Chapter 7 Section 7.3.1.

2.3 Collection of sediments

For the assessment of sediment quality, surface sediments are most commonly collected, but deeper sediments may need to be collected for evaluating the risks of activities such as dredging. Generally, the upper 10 cm of sediments will be occupied by 'epifaunal' (or 'epibenthic') and 'infaunal' organisms. Epibenthic species (those living at the sediment surface or just above it), such as shrimps and some amphipods, might only be exposed to contaminants in surficial sediments (0–1 cm), while others (such as bivalves, polychaetes) that are infaunal irrigators may be exposed primarily to contaminants at several centimetres depth. Determining contaminant concentrations in both the 0–2 cm and 2–10 cm depth sediments should provide sufficient information to assess major contaminant exposure pathways for most organisms.

A large range of devices is available for collecting sediments, and reviews of their uses and suitability for different collection conditions are available (Mudroch and Azcue, 1995; USEPA, 2001). The most important requirement for sediment collection devices is that they minimise changes to the integrity of the collected sediment, because substantial disruption of the sediment's structure will distort its chemical and physical characteristics. An example of possible disruption includes mixing of previously redox-stratified chemical substrates with layers of differing particle size and composition, thereby influencing the bioavailability of contaminants and the potential toxicity of the sediment (Simpson and Batley, 2003). Experience with the use of any sediment sampling apparatus is required to minimise such disruption.

The quantities of sediments that should be collected will depend on the analyses and tests to be undertaken. Generally, 1 kg of sediment from each site should be sufficient for analyses of most contaminants (for example, 500 g for elutriate tests, 250 g for organics, 50 g for metals and metalloids, 50 g for acid volatile sulfide (AVS), 50–200 g for analysis of particle size and other physical properties). All samples should be stored using equipment and techniques appropriate for the desired analysis (for example, glass jars for organics; sediments frozen for AVS) (see Chapter 3). In addition, 2–3 kg may be required for bioaccumulation or toxicity tests (for example, 0.1–1 kg/species; 4 × 200 g replicates), and these samples should be stored cold (not frozen).

Sample containers and sampling devices should be cleaned thoroughly before use by soaking plastic containers and devices in 10% nitric acid (for metals) or rinsing glass containers or devices with acetone for organics (ASTM, 2008; USEPA, 2001). Obviously, nitric acid is inappropriate where nitrogen forms are being analysed. Before sample retrieval, and between each sampling event, the outside of the sampling device should be rinsed clean with water from the sampling station. For some assessments, more rigorous between-sample cleaning of the sampler might be required (for example, washing with detergent to remove oil films, then further rinsing to remove excess detergent).

When sampling sediments of unknown composition, ASTM (2008) recommends that samplers and containers be subject to: (i) a soap and water wash, (ii) a distilled water rinse, (iii) an acetone or ethanol rinse, and (iv) a site-water rinse. Where the test sediments are expected to be very contaminated, reference sites should be sampled first, to minimise cross-contamination.

It is important to check the integrity of the collected sample before accepting it for subsequent physical, biological, chemical or toxicity testing. Grab samples are acceptable if the surface layer appears to be undisturbed (sediment–water interface is intact and relatively flat, with no sign of channelling or loss of fine materials), and if the volume of sediment is sufficient. For both grab and core sampling, several replicate samplings of a site may be required to obtain the desired quantity of material.

2.3.1 Collection of surface sediments

The surface layer provides information on the most recently deposited sediment materials and should be used to determine the horizontal variation in sediment properties and the distribution of contaminants. Knowledge of the bathymetry and distribution of physically different sediment types and habitats (for example, seagrass areas) may aid selection of sampling sites.

Grab samplers should generally be used to collect surface sediments, because of their ease of handling and operation and their versatility for collecting a range of sediment substrates. The Birge-Ekman sampler is suitable for sampling soft sediments in shallow

quiescent water, and small or lightweight designs may be operated by handline while wading or from a boat. The Van Veen grab sampler is more versatile for collecting sediments with a range of sediment properties, and is generally operated by winch from a boat. Importantly, the grab sampler should protect the sample from disturbance, minimise washout of fine-grained sediments and allow easy access to the surface layer by lifting of movable cover flaps. Both the Birge-Ekman and Van Veen samplers permit relatively non-disruptive sampling. During deployment of a grab sampler, the speed of descent should be controlled, with no 'free fall', so that a bow wave is not created that mixes or disperses the surface layer upon impact. Birge-Ekman samplers are not recommended for use in strong currents or high waves and may be less stable during sediment penetration. A discussion of these and other grab samplers (for example, Ponar, Petersen, Shipek, Smith-McIntyre) is available elsewhere (Mudroch and Azcue, 1995; USEPA, 2001). Grab samplers are preferred for the collection of all submerged surface sediment samples. If sediments are collected from areas exposed at low tide, a shovel or other hand implement may be appropriate.

2.3.2 Collection of sediments at depth

Sediments from depths greater than 15 cm below the sediment–water interface may be collected to determine the spatial (vertical and horizontal) variation in sediment properties and the distribution of contaminants. This is necessary when defining volumes of contaminated sediment for dredging or for investigating historical contamination. Three-dimensional mapping (for example, kriging) of contaminants may be used to identify zones of more highly contaminated materials (ASTM, 2010).

Core samplers should be used when assessments require: (i) accurate resolution of the depth of surficial sediments, (ii) detailed vertical profiles of sediment properties, contaminants or sedimentation history, and (iii) where it is important to maintain an oxygen-free environment (USEPA, 2001).

Hand corers can be used to collect sediment from <1 m sediment depth, by wading in shallow waters, or by divers. Vibrocorers yield excellent sample integrity and are recommended for the collection of deep cores (up to 6 m), or where sediment consists of very compacted or large-grained material (for example, gravel). Box corers (<1 m depth) are particularly useful for (i) collecting large volumes of sediment from a given depth (they allow sediment for all tests to be collected in one sample), and (ii) for collecting sediments for pore-water extraction and characterisation. For routine monitoring, the Phleger, Alpine, and Kajak-Brinkhurst corers may be more suitable. A discussion of the operation of these and other core samplers (for example, Alpine, Box, Gravity, Kajak-Brinkhurst, Phleger, Piston) is available elsewhere (Mudroch and Azcue, 1995; USEPA, 2001).

Hand corers are typically 60 cm long × 5–10 cm diameter and made from Perspex® or polycarbonate, desirably with a bevelled leading edge. For clay sediments, corers of, for example, >7 cm diameter are preferable, to reduce the friction of the clay within the core tube. This allows the sediment to pass more freely and results in less-compressed core samples (giving more accurate depth information). After immersion in the sediment, the tubes are capped with tight-fitting polyethylene (or other appropriately non-contaminating) caps, and as soon as they have been withdrawn from the sediment the bottoms are similarly capped. In water of less than a few metres depth, PVC core tubes up to 4 m in length can be immersed from a boat, and sectioned on shore to recover only the desired sample depth (<1 m). Perspex® corer tube designs with extendable aluminium pole sections can also be constructed for use from a boat in shallow waters, but they should be designed so that the cores can be easily extruded immediately following collection (USEPA, 2001).

Where measurement of fluxes of contaminants from sediments is an objective, 40 cm × 15 cm diameter Perspex® corer–reactors are ideal (Jung *et al.*, 2003). Here the corer, containing collected sediment, becomes a laboratory reactor with the addition of site water and a reactor head comprising stirrer, gas bubbler and sampling ports (see Chapter 3 Section 3.8.2).

Core samples are generally acceptable if the core was inserted vertically in the sediment and minimal disturbance and loss of sediment occurs during retrieval. To prevent mixing of sediment inside the core, care should be taken to keep the core upright and stationary during transport to the water surface and before sectioning. The entire space over the sediment in the core tube should be filled with site water, and both ends of the core tube capped and taped to prevent mixing of the sediment inside. If sediment oxidation is a concern (for example, changes in metal bioavailability or volatile substances), then the headspace of the core tube should be purged with an inert gas such as nitrogen or argon. Repeated sampling of a site (that is, several cores) may be necessary to obtain the desired quantity of material from a given depth. Records should be made of vertical stratification (for example, via photographs and a geological log that identifies profiles and records strata of interest).

2.3.3 Collection of suspended sediments

Suspended sediments are often collected from surface waters to determine the concentrations of particulate-associated contaminants as distinct from dissolved contaminants. Depending on their particle size, these sediments may remain in suspension, be transported depending on flow conditions, or settle to the bottom sediments. Methods used in collecting suspended contaminants depend largely on the questions being addressed. The commonly used methods have been reviewed in several publications (Ongley, 1996; Perks, 2014), and include:

- grab sampling,
- pump samplers,
- integrating samplers, and
- sediment traps.

For most sediment quality assessments, grab sampling will suffice and the sediment will be isolated by either filtration or centrifugation, with the rest of the sample usually being used for dissolved contaminant analysis. The sample size will need to be decided, to ensure that the volume of sediment is sufficient to allow the analytes of concern to be reliably quantified. If grain size is to be considered, larger sample volumes will be needed.

Sediment traps are a common means of collecting settling particles, providing a useful insight into the contaminant status of newly deposited sediments that are not easily assessed by sampling the surface layers of bottom sediments. Gardner (1980, 2000) has comprehensively evaluated the optimal design considerations for sediment traps and discussed how they are best used.

2.4 Field records

Records of field measurements and observations are important for any assessment of sediment quality. Field records should include site identifier (name or number), site location (recorded by GPS), time and date of sample collection, sample identifier (number or name) and replicate number. Depth should also be recorded if cores are sectioned in the field. Measurements should include (i) water quality parameters (pH, redox potential, dissolved

oxygen, temperature, conductivity/salinity, turbidity and water depth) in the water column 5–20 cm above the sampling site, and (ii) pH and redox potential of the surface sediments and depth sediments if sectioned from cores in the field. Observations should include (i) water column depth and conditions during sampling (tides, waves, clarity), (ii) sediment properties (gravel, sand, silty-sand, silt), the occurrence of debris (wood, shells, other debris) and plants (for example, seagrass), the sediment depth sampled, and the overlying water depth. Collected cores should be photographed, visually examined and any changes in strata with depth recorded (texture and consistency, colour, presence of biota or debris, evidence of oil). If the sediment colour changes from light brown to grey to black down the sediment core, the depth and thickness of these layers should be recorded; they may be evidence of differing redox conditions. Chain-of-custody forms should be prepared that identify each sample collected and the analyses to be conducted on the sample.

2.5 Field processing, transport and storage

Any form of disturbance to the sediments, whether through the act of sampling, field processing or transportation, will affect the bioavailability of the contaminants (Thomson *et al*., 1980; Bull and Williams, 2002; Langezaal *et al*., 2003; Simpson and Batley, 2003; Simpson *et al*., 2004). Although disturbances to the sediments cannot be eliminated, it is important that they are minimised. Following collection, sediment samples should be stored cold (on ice) to reduce loss of volatiles and decrease bacterial activity. Field processing, or manipulation, of sediments may result in changes in the speciation, and bioavailability, of substances by disruption of the equilibrium in the pore-water/sediment system. For example, sediment mixing or sub-sampling may cause intrusion of air into sediments and the oxidation of sensitive substances, changes in the oxidation states of previously redox-stratified sediment components ($Fe(II)/Fe(OH)_3/FeS$) and the subsequent reactions of these new phases, and changes to the availability of organic compounds by disruption of their equilibrium with organic carbon in the pore-water/sediment system. To minimise sediment oxidation the headspace above the sample should be purged with an inert gas such as nitrogen or argon, or the sample should be stored in an inert gas atmosphere. Filling containers completely will minimise the immediate interaction of samples with air.

Sediment samples to be analysed for metals or inorganic contaminants should be stored in plastic materials. High-density polyethylene (HDPE) or polytetrafluoroethylene (PTFE) containers are most suitable, although well sealed plastic bags may also be suitable (samples are generally double or triple bagged to avoid losses or contamination if one bag opens). Samples for organic contaminant analysis should be stored in borosilicate glass containers with PTFE lid liners (preferably brown glass for photo-reactive compounds such as PAHs). These materials will minimise leaching, dissolution and sorption (ASTM, 2008). Sub-samples should be collected away from the sides of the collection apparatus to avoid potential contamination. All utensils (for example, spoons, scoops, spatulas) that come in direct contact with sediment samples during handling and processing should be made of non-contaminating materials (for example, HDPE or PTFE for samples for metals analyses, and high-quality stainless steel for samples for organics analyses). All equipment and containers used to sub-sample and store sediments should be cleaned using appropriate techniques (ASTM, 2008).

Before sub-sampling from a grab sampler, the overlying water should be removed by slow siphoning using a clean tube near one side of the sampler. For sediment cores, the choice of depth horizon(s) and the techniques for sectioning of the core will depend on the study objectives as well as the nature of the substrate. Sectioning can be undertaken either

by splitting the core tube longitudinally or by extrusion through applying upward pressure on the sediment from the base (for example, using a Perspex® piston designed to fit snugly in the core tube). The exposed sediment should be immediately cut into sections of the desired thickness using a stainless steel or plastic (HDPE or PTFE) cutter. The outer layer of sediment that has been in contact with the cutting blade and the core tube (1–2 mm) may need to be removed and discarded to eliminate possible contamination before the sample is transferred to the storage container. Further discussion of techniques for sectioning cores is available elsewhere (Environment Canada, 1994; Mudroch and Azcue, 1995; USEPA, 2001). Depending on the tests to be made on the collected sediment, oxygen-free conditions may be necessary when the sediment within the core is extruded and processed (Simpson and Batley, 2003).

Sub-sampling is not easily done in the field, in which case cores should be chilled (on ice) or frozen, depending on the measurements to be undertaken. In either case, cores should be stored vertically, and undue agitation should be avoided during transportation because that will particularly mix the surface layers in unfrozen cores. Freezing has been found to rupture bacterial cells and release accumulated elements, such as selenium, into pore waters (Jung and Batley, 2004). Freezing of sediments is recommended if they are to be analysed for either AVS or total contaminants.

Maximum holding times and storage methods are governed by sediment type, contaminant characteristics, the expected use of the collected sediments or sediment components (for example, pore waters), and the tests to be undertaken on the sediments. The general recommendation is to store sediments and pore water in the dark at 4°C (Carr and Nipper, 2003; Geffard *et al*., 2004). Specific preservation requirements for metals, ammonia, cyanide and sulfide in pore-water analysis are given in Chapter 3. Samples for analyses of total metals can be held indefinitely, but changes to metal speciation will begin occurring within days of collection, as also will partitioning of contaminants between sediments and pore waters (Carr and Chapman, 1995; DeFoe and Ankley, 1998; Cole *et al*., 2000; Simpson and Batley, 2003). Changes in bacterial activity will cause changes to the concentrations of ammonia, sulfide, Fe(II) and biologically-active sediment components, particularly in pore waters. It is generally recommended that if pore waters are of interest, they should be extracted immediately after collection and subjected to appropriate preservation procedures, with suitable storage containers for each analyte (may need separate containers). Most extractable organics (for example, phthalates, organochlorine pesticides, polychlorinated biphenyls (PCBs), PAHs, hydrocarbons and dioxins) should be extracted from sediments within 14 days of sample collection, while 7 days should be the maximum storage length before extraction of samples for analyses of organic contaminants that are susceptible to losses due to volatility or microbial degradation. Storage containers for samples for analyses of organics (sediments or water extracts) should be glass, and plastic lids should be PTFE-lined to minimise adsorptive losses.

Sediments for use in toxicity tests should be tested as soon as possible after collection, but this interval will often be determined by the time required for chemical analyses. It is suggested that sediments should be stored for no longer than 8 weeks before toxicity testing (USEPA/USACE, 1998; Geffard *et al*., 2004). Longer storage times may be appropriate, depending on properties of the sediments and the concentrations and types of contaminants. Extended storage of sediments may result in: (i) losses of labile or volatile contaminants (for example, ammonia, volatile organics, AVS) or (ii) changes to the redox properties of the sediments because of increased or decreased bacterial activity (Simpson and Batley, 2003). Either of these processes may result in major changes to the concentrations or bioavailability of the

sediment contaminants. For sediments that are stored for long periods (for example, greater than 8 weeks), re-analysis of some contaminant concentrations may be required before toxicity testing.

2.6 Sediment manipulations prior to testing

Sediments are often manipulated in the field or laboratory before chemical or toxicity testing. Manipulation may involve sieving to remove large particles and debris, or the separation of indigenous biota, or homogenisation so that a large sample can be used for several chemical and biological tests (see, for example, Bufflap and Allen, 1995; Carr and Chapman, 1995; Sarda and Burton, 1995; Burgess and McKinney, 1997; Sijm *et al*., 1997; Chapman *et al*., 2002, Simpson and Batley, 2003; Fisher *et al*., 2004). Most manipulations of sediments will alter the properties of the sediments and affect contaminant bioavailability, and the effects of these on the test data need to be evaluated. All procedures used to prepare sediment samples for analyses and tests should aim to minimise disturbances and should be fully documented in reports.

It is desirable to undertake some assessment of how sample manipulation may affect the concentrations (for example, via loss of volatiles), bioavailability (for example, via changes to AVS; partitioning in pore waters) and toxicity of contaminants in the collected sediments. In freshly collected whole-sediment samples that have been minimally manipulated, later interpretation of bioavailability and toxicity test data will be aided by preliminary measurements of pH, redox potential, total organic carbon (TOC), AVS, iron and particle size distribution, and analyses of total and weakly-extractable contaminants and pore-water contaminants. If sediment samples undergo major manipulation (for example, sieving) or are stored for long periods before testing (for example, longer than 4 weeks), reanalysis is desirable for those parameters likely to be affected by these manipulations (for example, pH, AVS, pore waters, volatile organic compounds).

2.6.1 Sieving

Sieving of sediments causes major changes to sample integrity and possible losses of particular components (for example, volatile organics). Valid reasons for sieving sediments include:

- to remove coarse material (debris, rock, shells, wood >2 mm in diameter) that may interfere in analyses (only the <2 mm sediment fraction is used when comparing contaminant concentrations against guideline values);
- to obtain information on the distribution of contaminants in different sediment size fractions; or
- to remove indigenous organisms from the sediments before performing toxicity tests.

For toxicity tests, it is preferable that none of the test sediment samples be sieved. However, if test procedures require sieved sediment, then all of the test samples, including control and reference sediments, should be sieved. In some cases, indigenous organisms may be handpicked from the samples. Indigenous organisms may confound results of toxicity tests by being similar to test organisms or by preying on the test organisms. To remove indigenous organisms, the most appropriate procedure is press sieving, whereby the sediments are pressed through a chemically-inert (non-metal) sieve, using either a gloved hand or a plastic or Teflon®-coated spatula. Generally, 2 mm sieves should be sufficient for removal of most problematic macrofauna. As an alternative to sieving, organisms may be handpicked (using tweezers or forceps) from sediments that have been spread out in

a shallow tray. A record should be made of what is removed or retained on the sieve (for example, organisms, shells, gravel and other debris). Sediment samples that are to be used for toxicity testing should not be washed through sieves using water, because that will remove contaminants and alter bioavailability, and further processing is likely to be required to remove the excess water. Samples that are to be used for both chemical analysis and toxicity tests should be sieved together, homogenised, and then split for their respective analyses. Sieving (or handpicking) may need to be carried out in an oxygen-free atmosphere to minimise oxidation of sediment components.

Wet sieving of sediments is recommended when information is required on contaminant partitioning between different particle size fractions. Samples should be thoroughly homogenised before wet sieving is undertaken. Deionised water or clean seawater should be used to wash the sediment through a chemically-inert sieve material with the aid of a chemically-inert spatula. Sieves and spatulas should be high quality stainless steel when organics are the main contaminants of interest, or non-adsorbing plastics (for example, nylon, polyethylene, polypropylene, Teflon®) when metals are to be analysed. Generally the silt fraction of the sediments (approximately <63 μm) is considered to be the most important with regard to contaminant partitioning, especially for metals. Some redistribution of contaminants to the finer sediment fraction may be expected due to solubilisation of larger particles. If there is a concern about losses and redistributions of contaminants during sieving, the recommendation is to undertake only total contaminant analyses, with wet sieving used to determine the grain size distribution only on a separate sub-sample.

2.7 Sediment heterogeneity

As discussed earlier, sediments, unlike water, can be remarkably heterogeneous. Vertical stratification of contaminants frequently occurs in sediments because of varying historical inputs and natural layering of sediments from differing origins which have different contaminant-binding properties. Spatial heterogeneity both in grain size and in contaminant distribution has also been shown to involve micro-niches with high concentrations of contaminants, organic matter and microbial activity (Shuttleworth *et al.*, 1999; Robertson *et al.*, 2009; Stockdale *et al.*, 2009). These processes are superimposed on the normal layering of biogeochemical processes within sediments (for example, Fig. 2.1; see also Chapter 1 Fig. 1.3). It is well documented that organisms engage in bioturbation (burrowing) and bioirrigation (introducing overlying water into burrows) and that these affect the migration of sediment contaminants (Forster, 1996; Petersen *et al.*, 1998; Ciarelli *et al.*, 1999; Rasmussen *et al.*, 2000; Ciutat and Boudou, 2003; Simpson and Batley, 2003). The different feeding and burrowing behaviours of organisms affect how they sort particles, enrich or deplete organic matter, inject oxygen into localised sediments (Pischedda *et al.*, 2008; Volkenborn *et al.*, 2010) and alter contaminant fluxes from sediments (see Chapter 1 Fig. 1.2). Some of these complex issues may challenge our more simplistic view of sediment chemistry which underpins the sediment quality guideline values and their application, where sediments are considered as homogeneous and in some instances are homogenised before investigation. To resolve heterogeneity issues associated with defining contaminant concentrations within an area (for example, mean or 95% upper confidence limits), a systematic and statistical evaluation of potential outliers may be required (as discussed in Chapter 3 Section 3.4.1).

The effects of localised heterogeneity with respect to organic contaminants was illustrated by Guerrero *et al.* (2003) in a study of pyrene bioaccumulation by clams with various types of artificial sediments and a natural one. Variations in the sediment–water partition coefficient (K_d) of the particles defined the window of bioavailability for pyrene when it was

adsorbed on the surface of ingested sediments. However, the natural sediment tested did not fit easily into the partitioning interpretation. The main reason could be that K_d is really only meaningful for a single defined surface. In natural sediments there is a large range of components, each with a different partition coefficient, so that the averaged value that is measured experimentally needs to be interpreted carefully. This could be particularly true if the test organism is a selective feeder that may ingest components of the sediment with quite specific K_d values that differ considerably from those of the 'bulk' phase.

The microbial degradation of labile organic matter in bulk sediments determines the redox potential (Eh) and the pH observed at various depths in bulk sediment, and is responsible for a variety of secondary reactions involving metals (for example, desorption, release to pore water, fixation as sulfides). These redox reactions lead to vertical zonation of pH, Eh and various chemical species in sediments. Since the flux of labile organic matter to the sediments is usually much faster than the diffusive flux of oxygen across the sediment–water interface, it is commonly observed that oxygen drops to zero within a few millimetres of the sediment–water interface in productive sediments. The sub-oxic depth, dominated by Fe(III)-reduction, can be present at reasonable depths even in productive sediments. Sediments can be anoxic well above the depth to which most benthic animals burrow.

In addition, during the microbial degradation of organic matter in the sediments, reduced forms of the electron acceptors are produced and released to the pore waters. Some of these solutes, such as sulfide, are toxic to most benthic animals. The natural concentrations of sulfide found in pore water are not toxic to most invertebrates (Wang and Chapman, 1999). It is important that such dynamics are understood and the chemical changes that occur when such sediments are disrupted are appreciated when designing and conducting laboratory toxicity tests on field-collected sediments and on artificially-prepared test sediments. Lee *et al*. (2000), for example, documented how disruption of anaerobic sediment spiked with four metals shifted the exposure from ingestion of particulates (the primary route *in situ*) to pore-water exposure.

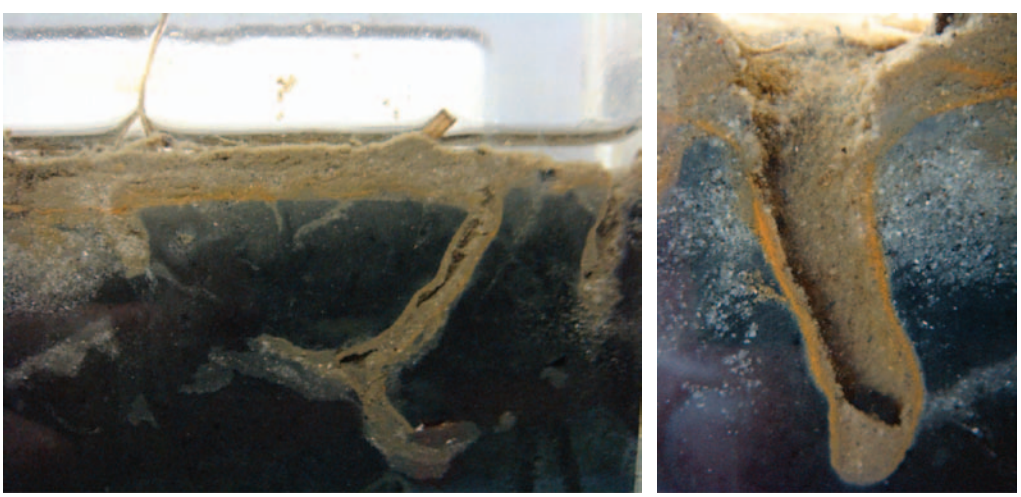

Figure 2.1. Photographs of cross-sections through the sediment–water interface and amphipod burrows showing the oxic (brown; here 5–10 mm depth at surface, 1–2 mm at burrow walls), sub-oxic (light to dark grey transition; here 5–10 mm depth below oxic surface strata, 2–5 mm beyond oxic strata from burrow walls) and anoxic strata (dark grey to black transition, beyond sub-oxic strata) (photos by David Robertson (2007), provided by Peter Teasdale, Griffith University).

2.8 Quality assurance/quality control (QA/QC) procedures for sediment collection and manipulation

Field replicates, field duplicates and field blanks are important components of all sediment assessment programs.

- Field replicates are two separate samples collected from the same location (same site and position) and analysed by the same laboratory to identify variations in sediments (for example, particle size, TOC and contaminant concentrations).
- Field duplicates are prepared from a single sample that is split in two and placed into two identical sample containers to assess variation in the sub-sampling and analytical methods. These differ from inter-laboratory comparisons, where duplicates are sent to different laboratories to identify possible variation due to analysis techniques (verification of primary laboratory).
- Field blanks (also used as sample handling blanks) are used to estimate the amount of contamination introduced during collection, transport and storage of the samples. For example, fine-grained clean sand or silt (with known contaminant concentrations) can be placed first in the sampling equipment, then transferred to the sample containers and transported to the laboratory and submitted as a sample.

These samples should be collected and handled in exactly the same way as the sediment samples and should be treated as blind samples so as to minimise bias in the analysis. For very volatile chemicals, spiking in the field may be a useful way of estimating losses occurring during transport and before testing. Chain-of-custody forms should accompany all samples from the time of collection through to reporting of analysis results.

2.9 Spiking of sediments with contaminants

The spiking of sediments with particular contaminants is undertaken: (i) to check recoveries of analytes (for QA/QC); (ii) to understand the partitioning of contaminants between sediments and water in different sediment matrices; (iii) to understand the transformation rates (degradation, volatilisation) of contaminants in different sediment matrices; and (iv) to quantify the effects of known concentrations of contaminants in toxicity tests (Chapter 4) or manipulated ecology experiments (for example, field-based recolonisation experiments, Chapter 7). Appendix B describes preparation of contaminant-spiked sediments.

Because sediment spiking will involve major manipulation of sediment properties, it is necessary to assess how the procedures will influence the sediment properties and contaminant bioavailability, in particular the partitioning between the dissolved and particulate phases (Northcott and Jones, 2000; Simpson *et al.*, 2004; Simpson and Batley, 2007). Thus, full descriptions of spiking procedures should be reported, with careful consideration of the necessary time required to achieve equilibrium with the sediments. It is recommended that the sediment parameters moisture content, pH, redox potential, organic carbon, AVS, particulate iron and manganese, and pore-water constituents (ammonia, sulfide, iron, metals) be measured before and after spiking so that losses or recoveries of added chemicals and the bioavailability of the added chemicals (or other chemicals present) can be adequately assessed. Failure to report these parameters will make it difficult to interpret results and assess contaminant exposure pathways and organism sensitivities (King *et al.*, 2006; Roman *et al.*, 2007; Simpson *et al.*, 2011; Besser *et al.*, 2013; Vandegehuchte *et al.*, 2013). Control sediment should undergo the same treatment as spiked sediments.

The most appropriate spiking technique will depend on the properties of the spiked contaminant (different metals and organic chemicals are influenced by different factors). For metals, generally the most important factor is an adequate period for equilibration. Oxidation of sediments during the disturbance introduced by the spiking process results in Fe(II) oxidation (of Fe(II) existing in pore waters or displaced from particles by added metals) with the release of H^+. An equilibration period of weeks to months may be required for the original redox potential to re-establish, and for pore-water metal concentrations to diminish as insoluble metal sulfides are formed. Detection of unrealistically low pH values or unnaturally high pore-water metal concentrations indicates that equilibrium has not yet been re-established (Simpson *et al*., 2004; Hutchins *et al*., 2009; Brumbaugh *et al*., 2013; Vandegehuchte *et al*., 2013). Dilution of a 'superspike' (a very high spiked concentration; also referred to as indirect spiking) has generally been found to result in the desired level of metal partitioning (low pore-water metals) being achieved more quickly (Hutchins *et al*., 2009; Brumbaugh *et al*., 2013).

For organics, equilibration times are often shorter than for metals (for example, days rather than weeks) (Landrum *et al*., 1992; Northcott and Jones, 2000). Here, a range of factors become very important considerations: namely, the hydrophobicity or solubility (often indicated from the K_{OW}, the octanol–water partition coefficient), choice of organic carrier solvent for spiking (solvent persistence, volatility, toxicity), and losses of chemical through non-target adsorption, degradation (UV light), transformation (hydrolysis) and volatilisation, along with the organic carbon content of the sediment (Ankley *et al*., 1994; Northcott and Jones, 2000; Fuchsman and Barber, 2000; OECD, 2007, 2010). Passive sampling techniques can be particularly useful for assessing the concentrations, bioavailability and equilibration of spiked organics (Sormunen *et al*., 2010; Section 2.12).

Generally, larger debris and indigenous organisms should be removed from sediments before spiking. Wet 'slurry' spiking techniques are recommended over dry spiking, as this will facilitate sediment–water interactions (Landrum *et al*., 1992; Northcott and Jones, 2000; USEPA, 2001; Simpson *et al*., 2004). Modification of sediment organic carbon will strongly affect the partitioning (and bioavailability) of organic chemicals (Di Toro *et al*., 2000; USEPA, 2012) and of metals (Besser *et al*., 2003; Strom *et al*., 2011); and modification of sediment sulfide concentrations (for example, AVS) will affect the partitioning of metals (Gonzalez, 1996; Leonard *et al*., 1999; Simpson *et al*., 2011).

Formulated control sediments have frequently been used for sediment-spiking studies, and procedures for their preparation are given elsewhere (Environment Canada, 1995; USEPA, 2001; OECD, 2007, 2010). The sources of materials used in the formulation can influence the contaminant binding phases (for example, organic carbon, AVS, percentage of silt) present in the formulated sediments, and should be carefully considered. The materials should be readily available, homogeneous, and have low contaminant concentrations and properties that do not change substantially upon storage or during use (that is, their concentrations of AVS or dissolved ammonia should be stable). The formulation should support the life-cycles (survival, growth and reproduction) of a wide range of benthic organisms.

2.10 General sediment quality parameters

There are several indirect stressors that modify sediment chemistry, thereby affecting contaminant bioavailability. Measurements should be made of pH, redox potential, moisture content, particle size distribution, TOC, AVS, particulate iron and manganese, pore-water

constituents (iron, manganese, ammonia, sulfide) and contaminants of concern in the various sediment fractions (total, weakly-extractable, pore water). The pH and redox potential measurements should be made in the field, on surface and depth sediments (the latter only if sectioned in field). It may be useful to repeat some measurements (for example, pH, redox potential, pore-water iron, AVS) before and after sample manipulation. In estuaries, pore-water salinity should be measured because it often differs significantly from the salinity of overlying waters (Chapman and Wang, 2001).

Important measurements of overlying water quality include pH, redox potential, dissolved oxygen, temperature, electrical conductivity/salinity, and turbidity at 5–20 cm above the sampling site. Measurements of total water depths, the amplitude of tides, and light penetration near the sediment–water interface will also aid data interpretation.

2.10.1 Sediment pH and redox potential

Sediment pore-water pH is the master variable controlling the speciation and bioavailability of metals. The water-quality guideline values for ammonia, cyanide and sulfide (all of which ionise as a function of pH) are pH-dependent (ANZECC/ARMCANZ, 2000b). The oxidation–reduction (redox) potential (Eh) is a useful indication (within limitations) (Teasdale *et al.*, 1998) of the biogeochemical condition of sediments, especially whether various substances are likely to be found in an oxidised or reduced state. Redox potential is an important control on sediment metal chemistry, particularly iron and manganese oxidation states and metal-sulfide chemistry of Ag, Cd, Cu, Fe, Hg, Mn, Ni, Pb and Zn. The oxic zone is typically a few millimetres below the sediment–water interface in productive sediments, underlain by a sub-oxic and then an anoxic area. The presence of acid volatile sulfides (FeS, MnS) is a buffer against metal release to pore waters because of their ability to exchange with soluble metals; this reaction will have a direct impact on bioavailability if the preferred uptake route is by exposure to pore waters. Although iron- and sulfate-reducing zones may overlap considerably, Fe(III)-reducing conditions often dominate in the Eh range from 0 to 200 mV and sulfate-reducing (sulfide-forming) conditions in the Eh range from –50 to –150 mV (Schüring *et al.*, 2000). Boundaries are difficult to define and are also dependent on pH (Stumm and Sulzberger, 1992). The pH- and redox-dependent equilibrium between Fe(II) in pore waters and the formation of Fe(III) hydroxide phases is very important in controlling concentrations of pore-water metal contaminants (Vink, 2002; Simpson and Batley, 2003). Both pH and redox potential measurements of pore waters provide useful process-related information on the nature of the sediments under investigation.

The measurement of sediment pH and redox potential are really measurements of the pore water rather than the sediment, and therefore need to be performed on wet sediment. Electrodes are available for both, but the act of insertion into the sediment may disturb the redox and pH profiles, so it is important to allow time for the equilibrium to re-establish before taking a measurement. The use of 'spear tip' pH (combination) electrodes that allow greater penetration into the sediments and less disturbance is the preferred approach for sediment pH measurements. Potentiometric measurements of redox potential should be made using a millivolt reader and, generally, a platinum electrode with combination Ag/AgCl or calomel reference electrodes (ASTM, 2014; APHA/AWWA/WEF, 2012). The millivolt reading should be reported as Eh versus the normal hydrogen electrode (NHE, also referred to as the standard hydrogen electrode (SHE)), and can be calculated from the measurement as follows:

$$Eh = E_{obs} + E_{ref}$$

where Eh = measured redox potential (mV) reported versus NHE, E_{obs} = observed redox potential for electrode pair used, and E_{ref} = redox potential of the reference electrode versus NHE.

The NHE is fragile and impractical to use directly. The combination platinum/reference electrode is usually calibrated against a redox standard solution to determine Eh, and then E_{obs} is calculated using the known E_{ref} for the standard. A range of redox standards are available: Light's solution (E *v.* NHE = 675 mV, at 25°C), Zobell's solution (428 mV), quinhydrone solutions (285 mV at pH 7, 462 mV at pH 4), and triiodide/iodide (420 mV at 25°C). As the redox potential will depend on the type of reference electrode used, the offset introduced must be compensated for. If, for example, a potential of 86 mV was obtained for the quinhydrone pH 7 redox buffer (at 25°C) (typical of E_{ref} *v.* Ag/AgCl), then use E_{ref} (= Eh – E_{obs} = 285 mV – 86 mV = 199 mV) as the offset. Each of these redox buffer solutions has a different storage life, ranging from hours to months. The use of Zobell's solution is recommended, but it is toxic and subject to oxidation (store in the dark). Inaccuracy, instability and poor reproducibility are common when measuring redox potential, resulting from disturbance of the sediment sample during insertion of the electrode. Details on preferred calibration solutions and how to clean indicator electrodes are provided by Teasdale *et al.* (1998). Acceptable error ranges for pH and redox potential measurements for sediments will be of the order ± 0.1 pH units and 20–40 mV respectively.

2.10.2 Water content

It is usual for measurements on sediments to be related to dry weight, but it is frequently preferable to undertake contaminant analyses on a wet sediment, because drying can alter the chemistry and in some instances result in losses of analyte. To convert these data to the preferred dry weight units it is therefore necessary to measure the water content.

Water content (often called moisture content) of wet sediment is determined gravimetrically by measuring the mass of water lost following drying at 110°C (Mudroch *et al.*, 1997). Before taking a sub-sample for moisture analysis, the sediment should be well mixed so that the water is evenly distributed throughout. The combined determination of sediment water content, density and porosity can be made according to the methods described in Mudroch *et al.* (1997). Sediment density and porosity are often useful parameters for describing sediment characteristics.

2.10.3 Particle size

Sediment particles (grains) generally range from sand, through silty sand and sandy-silt, to clays, although shells and other detritus may also be a significant proportion of many sediments (Table 2.1). The surface areas of these materials vary over orders of magnitude, and therefore so do the number of binding sites for metal and organic contaminants. Particle size often defines whether sediment is a good habitat for biota (for example, suitability for easy burrowing, or burrows not collapsing). Some species show preferences for sediments of particular particle sizes while others can happily survive in a range of particle sizes. Particle size will also influence benthic community structure. Fine sediments (for example, <63 μm) are typically those that are most heavily contaminated (greater surface area and more binding sites). A contaminant at a given bulk concentration in a sandy sediment will generally be more toxic than the same concentration in a silty sediment, because the partitioning to pore water will be greater. Because particle size influences both chemical and biological characteristics, it can be used to normalise chemical concentrations and account for some of the variability found in toxicity testing results and ecological

Table 2.1. Grain size classification of sediments[a]

Grain Size	Classification
<0.06 µm	Fine clay
0.06–0.63 µm	Medium clay
0.63–2 µm	Coarse clay
2–6.3 µm	Fine silt
6.3–20 µm	Medium silt
20–63 µm	Coarse silt
63 µm–2 mm	Sand
>2 mm	Gravel, coarse material, rocks, detritus

[a] Mudroch *et al.* (1997).

datasets (for example, biological assemblages). Particles <63 µm are more common in the gut of sediment-ingesting biota (Tessier *et al.*, 1984).

Sediment particle size analysis can be made by wet sieving, hydrometer or pipette methods or by laser particle size analysis (Mudroch *et al.*, 1997). For most assessments, it will be sufficient to determine just the fraction of sediment that is <63 µm (a standard sieve size), which will include the silt and clay fractions. Wet sieving is the recommended method, where deionised water (or clean seawater) is used to wash sediment through the sieve (see Section 2.6.1). The retained sediment is collected quantitatively and weighed following drying; this is done for each sediment size fraction if using multiple sieve sizes.

2.10.4 Total organic carbon

The forms of carbon in sediments may include elemental (for example, charcoal, coal, soot), inorganic (for example, carbonate minerals, shell debris) and organic (for example, wood debris, decomposed plants and animals, ash, and also hydrocarbon contributions from oils, tars and plastics) (Schumacher, 2002). In terms of analyses, Total Carbon = Inorganic Carbon + Organic Carbon. The total organic carbon (TOC) content of sediment is the sum of particulate organic carbon (POC) and dissolved organic carbon (DOC). Decaying detrital POC is distributed amongst mineral and amorphous particles in sediments. Inorganic particles are sites of bacterial activity and binding sites for both metal and organic contaminants. The binding of hydrophobic organic contaminants to different particulate phases will have quite different partition coefficients. In the ANZECC/ARMCANZ (2000b) guidelines, all organic contaminants are normalised to the TOC concentration of the sediment (that is, normalised to 1% TOC).

The TOC is the total amount of oxidisable organic material, and it will generally be measured using high temperature (for example, 1000–1500°C) dry combustion techniques where the combustion releases CO_2 which is quantified by titrimetric, gravimetric, manometric, spectrophotometric or gas chromatographic techniques, for example using instruments such as analysers of CHN (carbon, hydrogen, nitrogen) or TOC (Mudroch *et al.*, 1997; Schumacher, 2002). Chemical oxidation techniques (for example, dichromate oxidation) are not recommended because some organic compounds may not be analysed by these techniques. Inorganic carbon (for example, carbonates and bicarbonates) can be a significant proportion of the total carbon in some sediments. Therefore, analyses of TOC use samples that have been dried at 75–110°C following the removal of inorganic carbon

(by heating the sample with dilute acid until effervescence due to carbonates ceases). The methods generally have a limit of determination (LOD) of 100 mg/kg.

Another, less accurate, method of estimating sediment organic carbon content measures the 'loss-on-ignition' when a known mass of dried sediment is heated at ~400°C for 24 h. This is then followed by gravimetric analysis. However, a range of ignition temperatures are possible (temperatures from 350°C to 500°C have been used) which means that not just organic carbon but also other volatiles can be consumed or driven off; inorganic carbonates can also be lost at temperatures >440°C (Schumacher, 2002). Therefore, when the loss-on-ignition technique has been used, the report should make it clear that the measurement is an estimate of sediment organic matter, not TOC.

Black carbon (pyrogenic carbon or soot) has been shown to be important for binding hydrophobic organic contaminants (for example, PAHs) in sediments (Gustafsson *et al.*, 1997; USEPA, 2012). Black carbon is produced from the incomplete combustion of fossil fuels and vegetation. Examples of black carbon include charcoal (often remaining after bushfires) and coal dust (found near power stations or at coal ports), and these are often found in freshwater and estuarine sediments in Australia.

Hydrophobic organic contaminants are generally much more strongly associated with ('partitioned to') black carbon than other forms of natural organic matter. The differentiation of black carbon from other forms of carbon is usually made on the basis of the temperature of combustion. An oxidation temperature of 375°C has generally been found to provide a reasonable distinction between non-black carbon which is fully combusted below that temperature while black carbon remains and is not fully combusted until over 450°C (Gustafsson *et al.*, 2001). Black carbon measurements are recommended for assessments of sediments containing high concentrations of hydrophobic contaminants; they will assist in estimating partition coefficients used for predicting PAH bioaccumulation.

It is recommended that high temperature dry combustion techniques (for example, using CHN or TOC analysers) be used for analyses of POC where measurements are to be used to normalise organic contaminant concentrations to sediment TOC (as recommended in the ANZECC/ARMCANZ (2000b) guidelines). Loss-on-ignition measurements are useful when additional information is sought on relative differences in sediment organic carbon concentrations (for example, considerations for metal partitioning).

Note that the term 'blue carbon' refers to atmospherically-derived carbon (largely as carbon dioxide) that is sequestered by mangroves, salt marshes and seagrasses in sediments via leaves and roots (McLeod *et al.*, 2011).

2.11 Collection of pore water from sediments

Sediment pore water (or interstitial water) is defined as the water occupying the spaces between sediment particles. Typically pore water will occupy 30–80% of the volume of sediment, the volume being greater for fine-grained (silty) sediments than for sandy sediments. Water currents driven by surface water movements (for example, currents, tides, wind) or groundwater upwelling will influence pore-water composition and stability. In most depositional sediments, pore waters will be relatively static and it is expected that thermodynamic equilibrium will exist between contaminant concentrations in the pore water and in surrounding sediments. Sediment characteristics (for example, pH, organic carbon, sulfides, mineralogy and particle size) will greatly affect the partitioning of contaminants between the particles and pore waters (Di Toro *et al.*, 1991; Chapman *et al.*, 1998; Simpson and Batley, 2007).

Because many benthic organisms are in direct contact with sediment pore waters, this component of sediments is potentially a major exposure pathway. Accurate measurement of contaminant concentrations in sediment pore waters is therefore useful for assessing the potential bioavailability of contaminants. Pore waters are often isolated from the sediment matrix for toxicity testing with organisms that are sensitive to dissolved contaminants. The use of pore waters for toxicity assessment and toxicity identification evaluation (TIE) of sediment is discussed in Chapter 4.

Pore-water sampling, chemical and toxicity assessments are usually only undertaken on sediments for which total contaminant concentrations are above sediment quality guideline values (ANZECC/ARMCANZ, 2000b). Generally, pore-water assessments will not be necessary in sediments made up of coarse particles (sand, gravel) that have little binding capacity for sediment contaminants, nor in compacted clays that have little pore water with which organisms can interact. Where only chemical assessment is required, a range of techniques may be considered, including direct pore-water extraction and analysis, or passive sampling methods (which may be equilibrium techniques or kinetic techniques), or equilibrium partition calculations to predict pore-water concentrations based on the particulate concentrations.

Extraction of pore waters should be completed as soon as possible after sample collection. As already noted, sediments should not be frozen before pore-water analyses because that may potentially mobilise metals or metalloids through the rupturing of biological membranes (for example, cells of algae) (Section 2.5). Pore-water extractions from sediments should be conducted in an inert atmosphere, for example in a nitrogen-filled glove bag (or at least with minimal atmospheric contact), so that reduced species are minimally exposed to oxygen. Significant chemical changes can occur even when pore waters are stored for periods as short as 24 h (for example, Hulbert and Brindle, 1975; Sarda and Burton, 1995; Carr and Nipper, 2003; Simpson and Batley, 2003). Air exposure will result in the rapid oxidative precipitation of dissolved Fe(II) as Fe(III) hydroxide and slower oxidation of dissolved Mn(II) and sulfide. Following isolation from sediments, the pore waters should be stored so that oxidative changes, adsorption to containers or volatilisation are minimised (Carignan, 1984). Containers should be filled, with no headspace, to minimise changes in dissolved oxygen and contaminant bioavailability. Pore-water samples for chemical analyses should be preserved immediately, if appropriate (for example, acidification for metal analyses, frozen or preserved for pesticide or phenol analyses), or cooled to 4°C as soon as possible. Pore-water samples to be used for toxicity tests should be cooled to 4°C immediately after isolation and used in tests as soon possible. Storage containers should be appropriate to minimise adsorption or leaching of chemicals.

2.11.1 Pore-water sampling by centrifugation or squeezing techniques

A large variety of methods have been used for the isolation of pore waters from sediments (Carr and Nipper, 2003; Chapman *et al*., 2002). It is important to recognise that all methods have been shown to alter pore-water chemistry and affect the bioavailability and toxicity of metal contaminants (for example, Bufflap and Allen, 1995; Sarda and Burton, 1995; Chapman *et al*., 2002, Simpson and Batley, 2003). Pore waters will generally contain very low concentrations of dissolved oxygen and often have high concentrations of easily oxidisable species (for example, Fe(II)), and it is almost impossible to maintain these properties once pore waters are isolated from sediment (Simpson and Batley, 2003). Several good reviews are available that discuss pore-water sampling, precautions and artefacts (Carr and Nipper, 2003; Chapman *et al*., 2002).

Centrifugation or squeezing (*ex situ* extractions) will generally be the most useful methods for extracting pore waters for chemical analyses or toxicity testing. Centrifugation is the preferred laboratory method as it is a relatively simple procedure that allows rapid collection of large volumes. If exposure to oxygen is a concern, the sediments can be handled in a nitrogen-filled glove box or glove bag. With a bigger glove box, centrifugation can be undertaken in the box, although exposure can be minimised in the laboratory if centrifuge tubes are capped after purging with nitrogen in the glove bag. The centrifuge speed (and rotor radius) must be sufficient to create a relative centrifugal force (RCF) of 2000–5000 × g, and the centrifugation time needs to be chosen to achieve effective compression of the sediment and settling of particles to the sediment surface. For metal/metalloid analyses, it is desirable to use low-adsorption plastic containers (for example, Teflon®, HDPE, polycarbonate) and rapid extraction (for example, within 5–10 min), and to filter the isolated pore waters as soon as possible after separating and then preserve them to minimise changes in dissolved concentrations (Simpson and Batley, 2003). For analyses of organic contaminants, the solids should be removed by centrifugation (2000–5000 × g) using glass centrifuge bottles (for example, Corex, Corning®), and refrigeration (for example, 4°C) during centrifugation is desirable (to minimise adsorption and volatilisation). Longer centrifugation times can be used (for example, 20–90 min), and storage at 4°C in glass bottles with minimum headspace. For analyses of organic contaminants, pore waters should not be filtered following centrifugation as this can result in unacceptable losses. Dissolved organic carbon (DOC) analyses may also be made on these samples.

When pore waters are to be used for toxicity tests, filtration should generally be avoided because studies have shown that filtered samples generally have lower toxicity than unfiltered samples (Carr and Nipper, 2003). This is to be expected, because filtration procedures generally remove a larger proportion of fine or colloidal solids than do centrifugation or squeezing techniques. For accurately characterising sediment toxicity, colloidal material and fine particles present in the pore water may be of importance.

2.11.2 Pore-water peepers

For passive sampling, the most common early form of sampler is the peeper, in which solutes from adjacent pore waters diffuse across a membrane into compartments containing water (Hesslein, 1976; Carignan, 1984; Carignan *et al.*, 1985; Teasdale *et al.*, 2003; Brumbaugh *et al.*, 2013). Pore-water peepers are equilibrium dialysis samplers in which each chamber is filled with deoxygenated deionised water and covered with a fixed membrane (Teasdale *et al.*, 1995; Brumbaugh *et al.*, 2013) (Fig. 2.2). They can be single- or multi-chambered. The chambers are kept small to minimise disturbance to the redox gradients that influence the pore-water equilibrium and concentrations of dissolved metals in the surrounding pore water. When iron staining ($Fe(OH)_3$ precipitate) is observed in peepers, this may indicate that diffusion of dissolved oxygen from the overlying water into the pore water is being facilitated by the container's internal wall being in contact with both water and sediment.

Single-chamber peepers can be particularly useful for providing information on pore-water exposure during bioaccumulation and toxicity tests on sediment in the laboratory or in the field (Brumbaugh *et al.*, 2013). For this purpose, the peepers are positioned so they are completely submerged below the sediment–water interface (with only a small nylon cable tie protruding above the sediment surface). Single-chamber peepers are smaller than multi-chamber designs and cause less disturbance to localised redox conditions in pore waters near the membrane (Teasdale *et al.*, 1995; Doig and Liber, 2000). The peepers consist of laboratory low-density polyethylene (LDPE) snap-cap vials, with a hole punched in the

Figure 2.2. Photographs of single chamber (mini-peeper) (a, b) and multi-chamber (c) pore-water peepers (mini-peeper photos provided by William Brumbaugh, USGS).

cap and a polyethersulfone (PES) filter membrane inserted below the cap as the vial is sealed. To make 'mini'-peepers for use in laboratory sediment studies, use 2.5 mL vials with 0.45 μm pore-size and 25 mm diameter membrane (Fig. 2.2). Larger peepers for field deployment use 25 mL vials and a 47 mm diameter PES membrane.

Typically, multi-chambered devices (useful for obtaining depth profiles) can be made from a polymethylmethacrylate (acrylic) block ($8 \times 30 \times 1$ cm^3), into which are machined horizontal chambers 6.5 cm wide and around 4.2 mL in volume along its 30-cm length at intervals of 1 cm. The chambers are covered by a polysulfone membrane (on both sides), held in place by a thin acrylic cover sheet that is screwed into the block with nylon screws. The sheet has windows cut into it, to expose the chambers and their membrane windows. In use, these peepers are partially immersed in the sediments so that approximately one-quarter of their length is exposed in the overlying water, and they are left in place for at least 5 days (depending on the size of the chambers). When they are retrieved, the external surface is washed quickly to remove sediment particles and the membrane over each chamber is pierced with a micropipette and the water transferred to a suitable container and acidified for subsequent analysis. Chambers should be sampled in order from the bottom to the top so that anoxic samples have less time to be exposed to oxygen in the air.

The equilibration time of peepers and other *in situ* devices depends on their design (that is, the ratio of volume to surface area) (Teasdale *et al*., 1995; Davison *et al*., 2000). Thus a multi-chambered peeper of the type shown in Fig. 2.2 will equilibrate faster if it has windows on both sides rather than only on one side. (Much faster equilibration (for example, often 10 times faster than a peeper) is possible using diffusive equilibrium in thin films (DET), discussed in the next section.)

Multi-chamber peepers are suitable for obtaining low-resolution vertical distributions of pore-water contaminants in sediments (Carignan *et al*., 1985; Bufflap and Allen, 1995; Teasdale *et al*., 1995). The measurements in both the overlying water and at 1-cm intervals in the sediment pore waters yield a useful depth profile that reflects the effects of localised conditions of redox potential and pH on the partitioning of metals to pore waters (Teasdale *et al*., 2003). The concentration differences at the sediment–water interface can be used to calculate fluxes of metals to the overlying water.

One disadvantage of using peepers is that they take a relatively long time to reach equilibrium (several days to weeks) which increases opportunities for interference (natural or human). As with many trace metal techniques, there is considerable potential for error. Contamination from sediment particles is a major concern, so peeper users must be trained so they can obtain reproducible results. On recovery of the peeper from the sediment, anoxic pore waters will oxidise reasonably rapidly and so the chambers should be sampled without delay to avoid metals being removed as iron oxyhydroxide precipitates. Peepers generally produce insufficient volumes of pore water for toxicity testing purposes.

As with all passive samplers, considerable care is required to avoid inadvertent contamination of the device, and it is important to use preparation techniques suitable for sampling trace metals (for example, laminar flow cabinet, high purity deionised water, ultra-pure acids, acid-washing techniques, metal-free equipment for example, such as Teflon® or HDPE) when preparing peepers and handling samples.

2.12 Passive samplers

2.12.1 Diffusive equilibrium/gradients in thin films (DET/DGT) samplers for metals

The technique of diffusive equilibrium in thin films (DET) uses a thin hydrogel. Equilibration is reached much more rapidly than using peeper designs, allowing pore-water measurements to be made at higher resolution (Davison and Zhang, 1994; Harper *et al*., 1997). Complementary to DET is the technique of diffusive gradients in thin films (DGT), which uses a kinetic regime passive sampler that can provide *in situ* measurement of inorganic analyte concentrations in pore waters (or overlying waters) and fluxes from sediment pore waters (Zhang *et al*., 1995). In a DGT® device (registered trademark omitted hereafter), dissolved analyte species diffuse through a thin hydrogel layer (as used in DET) and become trapped in a gel typically impregnated with a chelating resin that selectively accumulates the metal of interest (or metalloid, using a specialised adsorbent). Analysis of DGT-accumulated metal has shown it consists of free metal ions, metal ions present as simple inorganic complexes, and labile organic complexes that dissociate over the time it takes to diffuse into the device (Zhang and Davison, 2000; van Leeuwen *et al*., 2005). DGT devices with different binding layers have been developed for measuring Cd, Co, Cr, Cu, Fe, Mn, Hg, Ni, Pb and Zn (Chelex-100 binding phase), Al, As(III, V), Hg, Mo(VI), Sb(V), S^{2-}, Se(IV, VI), V(V), W(VI) and U (using other binding phases) (Peijnenburg *et al*., 2014). The

major advantages of DET and DGT samplers over peepers are the short deployment times required (typically 8–48 h) and the greater resolution of pore-water depth profiles.

The DET/DGT devices (Fig. 2.3) can be purchased from DGT® Research, which also provides a detailed guide for the preparation and use of DGT samplers (DGT Research, 2015). The most common DGT device contains a layer of chelating resin (Chelex-100) separated from the test phase (for example, water, sediment or soil) by a polyacrylamide diffusion gel layer and a 0.45 μm membrane filter (Fig. 2.3). The resin strongly binds labile trace metal species that diffuse through the diffusive layer, creating a linear concentration gradient in the diffusive gel layer (Harper *et al*., 1998). When inserted into sediment, the accumulation of metals tends to locally deplete trace metal concentrations in the solution near the DGT probe, but resupply from the sediment solid phase can partially counterbalance this depletion. This is a 'kinetic regime' device, and so a linear relationship is assumed to exist between the accumulation of analytes in the DGT sampler and the deployment time.

In other words, during application of DGT, the removal of metals from sediment pore waters causes the concentration to decline immediately adjacent to the device. The DGT device causes a localised decline in pore-water metals that disturbs the dynamic equilibrium (partitioning) between pore water and sediment-bound metals and induces the release of metals into solution, the extent of which will depend on the rate of metal resupply (lability) from the sediment solid phase to the pore water (Harper *et al*., 1998). Release of metals from sediment particles to pore water (the DGT-induced metal flux) is likely to be more rapid for sediments that contain reactive forms of metals than for sediments that contain more inert forms of metals. Hence, differences in the DGT-induced metal fluxes (or the calculated pore-water concentration when assuming linear accumulation–time relationships) can provide useful information on the bioavailability of the metals in sediments (Roulier *et al*., 2008; Dabrin *et al*., 2012; Simpson *et al*., 2012; Amato *et al*., 2014). Peijnenburg *et al*. (2014) discuss recognised advantages and limitations of DGT measurements in sediments.

As for all trace metal sampling and analyses, it is important to avoid inadvertent contamination of the DGT device both before (for example, during acid-washing), during and

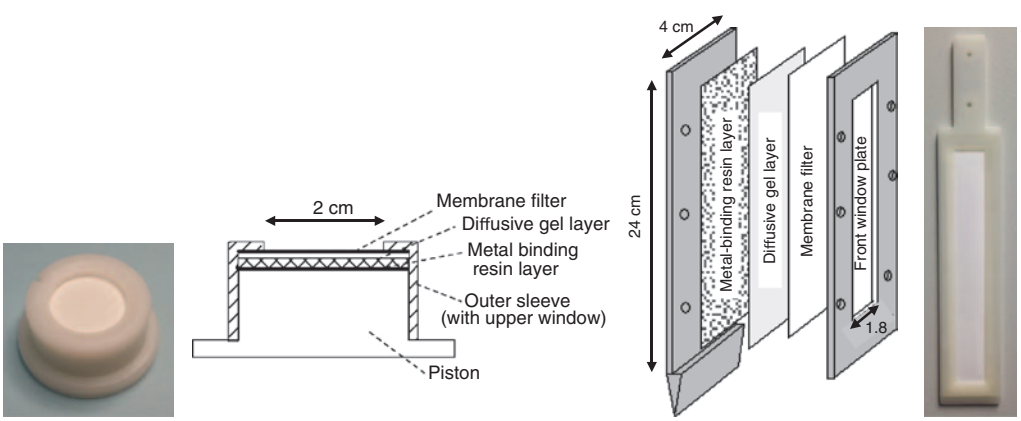

Figure 2.3. Examples of DGT samplers: (a) piston type (b, in cross-section); (c) planar type (exploded view). The concentration of metal measured by DGT (C_{DGT}, μg/L) can be calculated as $C_{DGT} = M\Delta g/DtA$, where Δg is the thickness of the diffusive gel and membrane (typically ≈0.09 cm, but this can be varied), D is the diffusion coefficient of free metal in the gel, t is the deployment time and A is the exposure area (A = 3.14 cm^2). (Modified from DTG Research, 2015.)

following deployment. When deploying and retrieving from sediments, (i) care should be taken to avoid adversely disturbing the sediment (for example, prevent the introduction of oxygen to sub-oxic or anoxic pore waters), (ii) the position of the sediment–water interface on the planar DGT sampler should be carefully recorded at the time of probe collection, (iii) observations such as of bubbles within the DGT sampler, torn membranes, or other anomalies should be recorded and the validity of these DGT results reconsidered.

After recovery from the sediment, the DGT device is disassembled, the resin layer is cut into appropriate slices (for sediment, sediment–water interface, overlying water) and the slices are immersed in a known volume of 1 M HNO_3 for at least 24 h to release the accumulated metals, before subsequent analysis. The mass of metal (M, in μg) accumulated in the resin gel is calculated from

$$M = C_e (V_{HNO3} + V_{gel}) / f_e,$$

where C_e represents the metal concentration in the eluent (μg/mL), V_{HNO3} is the volume of HNO_3 for the elution (1 mL), V_{gel} is the resin gel volume and f_e is the elution factor which is specific for each metal (typically 0.8).

The resin gel volume (V_{gel}, in mL) is calculated from

$$V_{gel} = l \cdot L \cdot \Delta r,$$

where l represents the width of the resin gel (typically 1.73 cm for the sediment probes available from DGT Research), L is the length of the resin gel (in cm) measured experimentally for each slice, and Δr is the thickness of the resin gel (typically 0.025 cm).

The flux of metal to the DGT device ($F_{DGT,}$ μg/m^2/h) is calculated from

$$F_{DGT} = 10,000 \cdot M / (t \cdot A),$$

where M is the amount of metal accumulated in the resin layer (μg), t represents the deployment time (h) and A is the surface area exposed to the overlying water (cm^2, equal to $l \cdot L$).

The time-averaged metal concentration at the interface between the diffusive gel layer and the overlying water (C_{DGT}, μg/L) is calculated from

$$C_{DGT} = F_{DGT} \cdot \Delta g / (D \cdot 1000),$$

where F_{DGT} is the flux of metal to the DGT device (μg/m^2/h), Δg is the thickness of the diffusive gel layer plus the membrane filter (for example, 0.064 cm), and D is the diffusion coefficient of the specific analyte in the DGT gel at the deployment temperature (for example, 0.0189 cm^2/h for copper at 19°C).

2.12.2 Passive samplers for organic contaminants

A variety of passive sampling methods are now available for *in situ* characterisation of pore-water concentrations of hydrophobic organic contaminants (HOCs) (and some quite polar compounds), and detailed reviews outlining advantages and limitations should be consulted to determine which is most appropriate for a specific assessment (USEPA 2012; Perron *et al.*, 2013a,b; Lydy *et al.*, 2014). These methods include semi-permeable membrane devices (SPMDs) that comprise low-density polyethylene (LDPE) tubing containing a high molecular weight synthetic lipid (triolein); polyethylene devices that consist of flat strips of LDPE but lack the triolein fluid used in SPMDs; solid phase micro-extraction (SPME) devices comprising fused silica fibres that are coated with a layer of absorbing polymer (for example, polydimethylsiloxane, PDMS); and polyoxymethylene devices which are similar to the polyethylene devices but comprise a harder polymer with greater sorption capacity

than PDMS. Standardised and commercially-available SPME methods are available for PAHs (ASTM, 2013).

Many of the considerations that apply to passive sampling devices for metals also apply to organic contaminants. For example, kinetic mode passive sampling devices may potentially deplete the concentration of the organic chemical in the pore water (that is, equilibrium is not maintained), resulting in the accumulated concentration representing a flux that is dependent on the sediment properties and the contaminant resupply rate (lability) from the sediments. The rate of exchange of hydrophobic organic contaminants from sediments to the sampler will depend on the characteristics of those contaminants, the sediment properties (particularly the concentration and form and diffusion properties of the particulate organic carbon), and the characteristics of the passive sampling device. Two main configurations of passive sampling device exist; they involve thin films or membranes cut into sheets or strips (including liquid or solids phases that accumulate hydrophobic organic contaminants), or coatings applied to fibres (in SPMEs for example) or surfaces (Lydy *et al.*, 2014), but the thicknesses (for example, of sheets) and dimensions can be modified for a range of purposes. The calibration method, the efficiency at which accumulation occurs, the choice of deployment time and the level of detection achievable vary considerably between the different devices and configurations. Some passive sampling devices can be deployed in either kinetic or equilibrium modes.

Quality assurance in relation to sample integrity, replication and repeatability is also very important. Ghosh *et al.* (2014) provide guidance for selecting, calibrating and implementing passive sampling devices for sediments. Lydy *et al.* (2014) provide a range of examples of the use of passive sampling devices for assessing bioaccumulation. Perron *et al.* (2013a) observed the following when several passive sampling devices were compared for monitoring estuarine waters:

- concentrations of PAHs were approximately three times greater using polyoxymethylene than when using polyethylene devices;
- concentrations of PCBs were approximately three times greater using polyethylene than when using polyoxymethylene; and
- SPMEs had inadequate detection limits for either PAHs or PCBs.

Such studies highlight the care required when using passive sampling devices for routine monitoring purposes. Even greater care is required when interpreting results for sediments or for emerging contaminants, such as polybrominated diphenyl ethers (PBDEs) and triclosan (Perron *et al.*, 2013b).

2.13 Preparation of sediment elutriates

Sediment elutriates are commonly used for assessing the effects of dredging operations on water quality (USEPA/USACE, 1998; NAGD, 2009). Elutriate tests are used to approximate the concentrations of contaminants that might be released from sediments that are disturbed or undergo unconfined disposal within waterbodies. The data analysis should consider the initial dilution, which in a sea-dumping context (for dredged material) is defined as mixing which occurs within 4 h of disposal. This dilution will depend on several factors, such as depth, layering in the water column, and current velocities and directions. Within the ocean disposal framework, typically a dilution factor of 100 is applied before comparison of the elutriate concentration with water quality guidelines (NAGD, 2009), but this factor can be refined using hydrodynamic modelling. Elutriates are often also prepared

and used for analyses where it is not possible to obtain sufficient pore water from a sediment to enable detection of particular analytes, for example, tributyltin or trace organic contaminants such as PCBs and some pesticides. Biological tests on elutriates in place of pore waters are not recommended, owing to the considerable changes in composition that occur following preparation and storage without preservation (as is necessary for toxicity testing) (see Chapter 4 Section 4.3.1) Elutriate manipulations are also applicable to any situation where the re-suspension of sediment-bound toxicants is of concern, such as during bioturbation and storms that might disturb sediments and affect water quality.

Elutriate procedures should suit the intended study. However, the general method for elutriate preparation involves combining water and sediment in a ratio of 4 parts water to 1 part sediment (by volume) and shaking the mixture end-over-end for 1 h (USEPA/USACE, 1998). After the 1-h mixing period, the mixture is allowed to settle for 1 h. The supernatant is then siphoned off and centrifuged to remove particulates before chemical analysis. As already noted (Section 2.11.1), filtration should be avoided when using elutriate waters for toxicity tests. Re-centrifuging elutriates may be a better alternative than filtration. If filtration is necessary, filters should be pre-treated (cleaned, soaked) and the first 10 mL of elutriate to pass through the filter should be discarded (Environment Canada, 1994). The dissolved or colloidal contaminant retained (adsorbed) by the filter may require analysis. Elutriates should be analysed or used in biological tests as soon as possible after preparation. If the elutriate needs to be stored, the storage period should be no longer than 24 h and storage should be at 4°C with minimum headspace in the storage container (Geffard *et al*., 2004).

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3

Chemistry of sediment contaminants

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3.1 Introduction

Chemical analysis of contaminants in sediments and associated pore waters provides one of the pivotal lines of evidence for the assessment of sediment quality. While in the past the focus has typically been on total contaminant concentrations, the quality of the chemistry assessment can be significantly enhanced by the use of measures that probe the chemical forms, partitioning and bioavailability of contaminants. This chapter discusses the current best practice approaches to the chemistry line of evidence.

3.2 Particulate metals, organometals and inorganics

Total particulate metal (and metalloid) analyses are best undertaken on an aliquot of sediment that was homogenised when moist, then dried before analysis. Note that for total analyses, samples should be sieved to remove particles >2 mm (see Chapter 2 Section 2.6.1) because guideline values for sediment contaminants typically exclude the coarse sand and larger debris. It is normally necessary to solubilise the metals by digestion in a concentrated acidic medium to extract a high proportion of the total concentration. Digestion of the sample can be microwave-assisted using a mixture of nitric, hydrofluoric and hydrochloric acids (in various combinations) (for example, USEPA, 2007a). As most of these analyses are not complete/exhaustive extractions, they are frequently referred to as total recoverable metal (TRM) analyses. Analyses of the diluted digests can be undertaken most conveniently using inductively coupled plasma atomic emission spectrometry (ICPAES) or inductively coupled plasma mass spectrometry (ICPMS). Hydride-generation ICPMS (HG-ICPMS) or hydride-generation atomic fluorescence spectrometry (HG-AFS) is usually required for As, Sb and Se, and cold-vapour atomic absorption spectrometry (AAS) or AFS for mercury. Easily achievable limits of determination (LODs) are shown in Table 3.1. Concentrations of iron and manganese should also be reported if the results are to be related to metal partitioning in sediments. Samples for metal and metalloid analyses can potentially be held indefinitely (that is, dried, freeze-dried or frozen) (note that drying is not appropriate if speciation analysis is necessary), but the suggested maximum holding times for most metal and metalloid analyses is 12 months. Holding times should be shorter for analytes where speciation is important, for example, 1 month for mercury and Cr(VI)

Table 3.1. Typical limits of determination (LOD) for common contaminants in sediments

Sediment contaminant	LOD	Unit
Ag, Al, As, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, Sb, Se, V, Zn	0.01 – 1	mg/kg
Mercury	1	µg/kg
Methylmercury	0.1	µg/kg
Tributyltin (TBT)	0.5	µg Sn/kg
Polycyclic aromatic hydrocarbons (PAHs) - individual	0.5–5	µg/kg
Sum of polycyclic aromatic hydrocarbons (total PAHs)	100	µg/kg
Total petroleum hydrocarbons (TPHs) (C6–C40)	10–100	mg/kg
Benzene, toluene, ethylbenzene, xylene (BTEX)	0.2	mg/kg
Phenols/phenolic compounds	0.1–2	mg/kg
Organochlorine pesticides (e.g. DDD, DDT, aldrin, chlordane)	0.2–1	µg/kg
Organophosphate pesticides (e.g. chlorpyrifos, diazinon)	5–10	µg/kg
Total polychlorinated biphenyls (PCBs)	5	µg/kg
Dioxins	0.0005–0.01	µg TEQ/kg[a]
Synthetic pyrethroids (e.g. bifenthrin, permethrin)	0.02–0.05	mg/kg
Phenoxy-acid herbicides (e.g. dicamba, 2,4,5-T)	0.1	mg/kg
Phthalates (e.g. dimethyl phthalate)	0.5–0.2	mg/kg
Carbamates (e.g. aldicarb, methiocarb)	0.02–0.05	mg/kg
Herbicides/fungicides (e.g. bromoxynil, glyphosate)	0.02–0.5	mg/kg
Dioxins	0.0005–0.01	µg/kg
Other analytes		
Total organic carbon (TOC)	0.02	%
Ammonia	0.2	mg/kg
NO_x	0.1	mg/kg
Orthophosphate	0.1	mg/kg
Cyanide	0.1	mg/kg

[a] TEQ = total equivalents; used for the toxicity weighted masses of mixtures of dioxins.

in sediments. Analyses for cyanide are typically undertaken using distillation methods, and the maximum holding time for sediments for this analysis is 14 days. Once the contaminant of interest has been extracted, the holding time for the extract will vary depending on the preservation method.

3.3 Particulate organics

For many organic contaminants at environmental concentrations, analysis is a highly specialised activity. This applies particularly for many of the newer pesticides, as well as for dioxins and chemicals used in antifouling paints, pharmaceuticals and personal-care products. Most organic analysis methods involve a pre-concentration step (typically, accelerated solvent extraction (ASE) or solid phase adsorption) followed by separation (liquid or gas chromatography) and quantification. Compound-specific detection is usually achieved by using coupled mass spectrometry or some other specific detector. Sample clean-up is required for certain analytes; for example, between high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) steps. The USEPA guidance document SE-846 'Test methods for evaluating solid waste' (USEPA, 2007b) provides broad guidance for analyses of organic analytes, including considerations of container types, preservation techniques and holding time for various volatile and semi-volatile organics. Maximum holding times for most organic analyses should be 14 days, although less than 7 days is desirable. Many laboratories use in-house methods developed for a specific suite of organic contaminants. Guidance on sampling, storage and analysis should be sought directly from the analytical laboratory. It is recommended that total organic carbon (TOC) analyses be undertaken for the normalisation of organic contaminant concentrations to 1% TOC.

Oils and greases include compounds such as hydrocarbons, vegetable oils, animal fats, waxes, soaps and other greases. Oil and grease concentrations are operationally defined depending on the solvent and the analytical method used. Petroleum hydrocarbons are constituents of oil and grease that remain in solution after contact with silica gel, and may contain both aromatic hydrocarbons (for example, polycyclic aromatic hydrocarbons (PAHs), and benzene, toluene, ethylbenzene and xylene (BTEX compounds)) and aliphatic hydrocarbons (methane, propane and kerosene). PAHs are the most toxic compounds found in petroleum products.

3.4 Quality assurance

For all analyses, a high degree of rigour is required to avoid sample contamination or contaminant losses, and to provide accurate results. Analyses of method/laboratory blanks, replicated extraction of samples and duplicate analyses of the same extract should each typically represent at least 5% of samples (often 10% for duplicates). These differ from the field blanks, replicates and duplicates described earlier (see Chapter 2 Section 2.8).

- Method/laboratory blanks are those prepared by the laboratory to assist in identifying any contamination introduced during analyses, including possible cross-contamination of samples or contamination during extraction or analysis. Analysis of method/laboratory blank samples is used to determine the detection limit and limit of reporting for a particular contaminant.
- Replicate and duplicate analyses are used to assess the precision of analysis performed by the laboratory. Replicate extractions are performed on an intra-laboratory split of the sample material. Duplicate analyses are performed on the extraction from a single sample. Usually a relative percentage difference (RPD) is calculated based on the percentage difference between the two measured concentrations from the duplicate analyses for a specific contaminant.

For metals and metalloids, both certified reference materials (CRMs) (also called 'standard reference materials') and matrix spike-recoveries should be analysed as a check of analytical accuracy. Analysis of CRMs verifies accuracy and precision in relation to results achieved by other world-class laboratories. A range of organisations can provide CRMs, and these are some of the major ones: Environment Canada; the European Institute for Reference Materials and Measurements (IRMM) which provides reference materials under the names BCR® and European Reference Materials (ERM®); the National Institute of Standards and Technology (NIST) in USA; the National Research Council of Canada (NRC); and International Atomic Energy Agency (IAEA) Reference Materials.

Matrix spike-recoveries assess potential losses due to handling and interferences (for example, due to particle size, or oils) that may affect extraction recoveries, and they also indicate whether 'matrix-matched' standards are sufficient for calibrating analytical instruments prior to sample analyses; that is, there is no suppression or enhancement due to differences in standard and sample matrices. For metals and metalloids, recovery limits of 80–120% (or closer, for example, 85–115%) are generally considered acceptable. For organic compounds, surrogate spikes are analysed. These are spikes of known amounts of several organic compounds similar in composition to the target analyte but not likely to be present within the sample. The percentage recovery result provides an indication of the ability of the method/laboratory to extract different organic contaminant types from the sample matrix. Recovery limits of 75–125% are generally acceptable for organic compound surrogate spikes.

Detection limits (DL) (three times the standard deviation of the blank for the specific analyte), limits of determination (LODs), limits of reporting (LORs) and practical quantification limits (PQLs) are frequently quoted when planning and reporting analyses. The terms LOD, LOR and PQL are analogous and often used interchangeably (generally being three times the detection limit or 10 times the standard deviation of the blank for the specific analyte). The LODs for metals, metalloids and organic contaminants vary greatly and are often dependent on the properties of the sediments (for example, mineral types) and the presence of other contaminants (for example, oils). Table 3.1 lists achievable detection limits for most common contaminants present in sediments.

3.4.1 Statistical analysis of environmental data: Outliers, non-detects and 95% UCLs

Environmental datasets created by randomly sampling a prescribed area are frequently skewed and are better represented by log normal or gamma distributions, than by a normal distribution (USEPA, 2013). These datasets may contain outliers and values below detection limits (non-detects) that can lead to incorrect decisions associated with the data analysis if not considered appropriately. The occurrence of 'non-detects' is common unless an environment is highly contaminated. There are several approaches to dealing with non-detects, including: (i) treating the observations as 'missing'; (ii) treating the observations as zero; (iii) using the numerical value of the detection limit; and (iv) using the numerical value of half the detection limit (ANZECC/ARMCANZ, 2000a). Sediment heterogeneity occurs naturally, and contaminants and the many factors affecting bioavailability (for example, silt, TOC, acid volatile sulfide (AVS)) and the distribution of organisms can be highly variable. A consequence of natural heterogeneity is that some measurements considered to be potential outliers may actually be strongly representative of the contaminated locations and therefore should not be excluded from the statistical description of the study area. However, as non-detects and outliers in a dataset tend to yield distorted (incorrect and misleading) values of the common decision-making statistics (for example, 95% UCLs,

which are the 95% upper confidence limits of concentrations within an assessment area), elevated outliers may need to be removed from datasets before these statistics are estimated. When many of the environmental data are non-detects, most approaches will result in the sample variance being severely underestimated.

In the absence of more sophisticated tools for analysing censored data, it is suggested that means and percentiles be computed using the full dataset with non-detects replaced by either the detection limit or half the detection limit (ANZECC/ARMCANZ, 2000a). Many statistical methods are now able to deal with datasets with or without non-detects and with or without outliers, but the proper disposition of outliers may also require best professional judgement. It is often useful to compute decision statistics with and without the outliers to evaluate their influence on the decision-making statistics. Quantile-quantile (q-q) and box-and-whisker plots may be very useful for visualising outliers within datasets. Statistical software such as ProUCL 5.0 (USEPA, 2013) provides a means to compute rigorous statistics from most environmental data, enabling well-founded decisions about contaminated sites. ProUCL currently uses classical outlier tests (Dixon and Rosner tests), but there are also other potentially more robust outlier identification methods available using other statistical software. Along with being free of charge and easy to use, a major strength of ProUCL is that it calculates and clearly reports 95% UCLs using a wide range of techniques, and it provides graphical outputs and recommends the most appropriate method and decision statistic based on the properties of the data used (number of data points and distribution), as tested by the developers of ProUCL using a large range of environmental datasets.

3.5 Contaminants in pore waters

Sediment pore waters, also referred to as interstitial waters, may be collected for a range of purposes, including for direct comparison to water quality guidelines (no specific guidelines exist for pore waters), for assessing contaminant fluxes from sediments, for assessing contaminant exposure routes and for toxicity testing or toxicity identification evaluation (TIE) studies (see Chapter 4 Section 4.10). Ability to collect sufficient pore waters is frequently a limitation for some of these purposes, restricting analytes to those that have adequate detection limits on small volumes (for example, 2–10 mL), or preventing use for toxicity assessment. The chemistry of pore waters can change rapidly following extraction (for example, through oxidation of Fe(II), and volatilisation of ammonia and organic contaminants), and analysis of most contaminants in pore waters is a highly specialised activity, particularly when dealing with saline waters.

3.5.1 Metals and metalloids in pore waters

Analyses of pore-water metals at guideline concentrations are achievable by several techniques for freshwaters, but there are substantially fewer techniques for saline waters. Analysis of metals and metalloids in saline samples requires specialist skills because of the extensive interferences that can result from the high salt concentrations in the samples. Spectrometric analyses often involve a matrix separation (in order to avoid interferences from the saline matrix) and a pre-concentration step. These are normally simultaneous procedures, such as chelation or solvent extraction, or isolation using chelating ion exchange resins. High reagent purity and appropriately clean work spaces (clean rooms or laminar-flow hoods), and 'clean' techniques are vital in order to attain accurate results, and particularly in relation to detection limits below 1 µg/L for metals in fresh and saline waters.

Direct analysis of metals in saline waters is possible for concentrations greater than 1 µg/L using combinations of ICPAES and ICPMS (with salt interference minimised by dilution or by the use of reaction or collision cells). Laboratories frequently dilute samples with deionised water (for example, 10- to 100-fold) to allow analysis by ICPMS, but after adjusting for such dilutions, the levels of reporting may increase by a similar magnitude. Detection limits are typically 10 times poorer than attained for freshwaters but may be suitable for monitoring some metals to the specified guideline value concentrations (for example, for sediment elutriates). Vapour-generation methods coupled with ICPMS, AFS (atomic fluorescence spectrometry) or AAS are used to determine As, Sb, Hg and Se in both fresh and saline matrices.

The measurement of dissolved metal or metalloid concentrations in sediment pore waters and comparison to water quality guidelines (WQGs) is useful for assessing the risk posed by these contaminants. The pore-water measurements assess only the potential for impact on biota through exposure to dissolved contaminants and do not assess dietary exposure to sediment or food particles. However, the pore-water concentrations are expected to be strongly influenced by the lability of metals bound to sediments, and in some cases useful relationships between pore-water metal concentrations and toxicity may exist even if the dietary exposure route is significant (Simpson *et al.*, 2012; Peijnenburg *et al.*, 2014).

Changes in pore-water metal concentrations, and inaccurate measurements, frequently occur due to non-ideal pore-water collection, preservation and analysis techniques. It is not possible to completely avoid disturbances to sediments during collection, and any disturbance has the potential to bring about major changes to metal partitioning between pore waters and sediment phases (increases or decreases) (Simpson and Batley, 2003). Pore-water metal concentrations are also expected to change with time, if sediments are collected then stored before isolation and preservation of pore waters.

A major factor influencing metal partitioning in sediments is the presence of dissolved oxygen. The redox conditions in sediments are usually stratified and zones are frequently referred to as oxic, sub-oxic, and anoxic. In undisturbed sediments, dissolved oxygen penetration decreases with depth. Dissolved oxygen may only penetrate to a few millimetres depth (for example, 2–10 mm) in sediments with high concentrations of organic carbon, but potentially to >10 cm depth in porous sandy sediments with low concentrations of organic carbon. This oxic layer overlies a sub-oxic zone dominated by Fe(II) and Mn(II), which itself overlies the anoxic zone where sulfide dominates (Jørgensen and Revsbech, 1985; Kristensen, 2000). Changes from anoxic to sub-oxic to oxic conditions significantly alter the partitioning and speciation of many metals.

During sampling and pore-water extractions using centrifugation or squeezing techniques, some disturbance through mixing of the redox-stratified layers is expected, but must be minimised where possible. Within the sub-oxic zone, pore-water Fe(II) concentrations are often in the 1–20 mg/L range and introduction of oxygen will cause rapid oxidation and precipitation of Fe(III) hydroxide, which can rapidly result in co-precipitation of metals and metalloids from the pore water. Thus pore waters are isolated from sediments in an oxygen-free atmosphere (for example, under nitrogen), and rapidly filtered and acidified following isolation to preserve the metal concentrations (see Chapter 2 Section 2.11).

Metal speciation in pore waters

Metals exist in natural waters in a variety of chemical forms or species, including: being associated with particles or colloids; as complexes with natural organic matter (for example, humic and fulvic acids) or synthetic organic ligands (for example, EDTA); as simple inorganic complexes (for example, with carbonate, chloride, hydroxide); and as the free metal ion. The objective is to measure the physico-chemical forms of a contaminant which

actually contribute towards toxicity. Contaminants associated with colloids and particles are assumed to have low potential toxicity, as also are many metal complexes that form with natural dissolved organic matter (a constituent of all surface waters). Metal toxicity is assumed to be related to the concentration of simple inorganic metal species. Applicable speciation methods are therefore the ones able to discriminate simple inorganic metal species from colloids, particles and/or complexes with natural organic matter.

Information on the form and potential bioavailability of pore-water metals such as Cd, Co, Cu, Ni, Pb and Zn can be derived from Chelex-labile metal measurements (Bowles *et al*., 2006; Simpson *et al*., 2014). Greater metal lability usually results in greater metal bioavailability. The method uses Chelex-100, a styrene divinylbenzene copolymer with iminodiacetic acid functional groups, which forms strong complexes with many metals. Any free metal ions or weakly bound metal complexes (termed 'labile metals') will bind to the resin. Labile metals include most inorganic complexes (for example, Cu^{2+}, $CuCl^{+}$, $CuCO_3$, $CuSO_4$, etc.) or weak organic complexes (for example, copper acetate) that dissociate rapidly and are complexed by the Chelex-100 resin. Typical non-labile dissolved metal complexes (for example, strong Cu-humic, Cu-fulvic, Cu-EDTA complexes) and colloidal forms will not bind and are considered to not be bioavailable.

Colloids have sizes in the range of ~0.001–1 µm (Wilkinson and Lead, 2007). It is well recognised that the filtration of waters through a 0.45 µm filter (that is, the filter size used to remove particles and most organisms – noting that 0.22 µm filters are frequently used to remove most bacteria) does not completely separate dissolved and colloidal forms of metals (Wilkinson and Lead, 2007; van Leeuwen and Buffle, 2009). After filtering through 0.45 µm filters, water samples may be filtered through a 0.025 µm filter to provide information on the fraction of 0.45 µm filterable metals that may be present in colloidal forms. While colloids of even smaller size may exist, it is not easy to use smaller filter sizes. Dialysis techniques are an alternative, but require longer durations for diffusion across the membrane which may result in undesirable changes in dissolved and colloidal metal forms. Results may be influenced by large charged particles such as proteins, if present, which cannot physically cross the membrane and prevent some other ions crossing due to charge stabilisation (termed the 'Donnan effect') (Ure and Davidson, 1995).

A further form of speciation analysis involves the discrimination between different oxidation states. Separate guideline values are given for each of the two oxidation states of arsenic and chromium (ANZECC/ARMCANZ, 2000b). This is because in each case there are marked differences in toxicity between these oxidation states: As(III) generally is more toxic than As(V), and Cr(VI) is more toxic than Cr(III). Analytical methods exist for speciation of As(III/V), Se(IV/VI), and Cr(III/VI) in waters and they can be applied to pore waters where sufficient volumes can be extracted and suitably preserved. Methods for arsenic speciation involve separation, hydride generation and detection by some form of atomic spectrometry. Chromium speciation has been determined by a variety of methods including ion exchange separations, selective co-precipitation and determination by graphite furnace atomic absorption spectrometry (GFAAS), ICPAES or ICPMS. The DGT technique described in Chapter 2 Section 2.12 also permits the speciation of As(III/V) and Se(IV/VI) in waters and may potentially be applied to pore waters using ferrihydrite, Metsorb (TiO_2) or 3-mercaptopropyl-functionalised silica gel binding phases (Luo *et al*., 2010; Bennett *et al*., 2011; Panther *et al*., 2013; Price *et al*., 2013; Peijnenburg *et al*., 2014). Generally, however, in order to avoid unnecessary analytical costs, the pragmatic approach is to first measure total arsenic or total chromium concentrations and compare them to the guideline values for the most toxic oxidation state (As(III) and Cr(VI)). If these values are exceeded, then specific determinations of each oxidation state should be undertaken.

3.5.2 Organic contaminants in pore waters

The measurement of dissolved organic contaminant concentrations in sediment pore waters is useful for direct comparison to water quality guideline values, for validation of equilibrium partitioning calculations (see Section 3.5.3 below), and for predicting the risk that contaminants pose for bioaccumulation and toxicity (Di Toro *et al*., 2000; Di Toro and McGrath, 2000; Hawthorne *et al*., 2007; USEPA, 2012). However, the dissolved concentrations of hydrophobic organic contaminants are typically extremely low because of the strong partitioning to sediments. The challenges associated with collection (for example, losses due to degradation, volatilisation) and analysis of pore-water organics are well documented (Carr and Nipper, 2003).

Organic analyses in waters are typically made by gas chromatography/flame ionisation detector (GC/FID) or gas chromatography/mass spectrometry (GC/MS) following solid phase extraction (USEPA, 2007b). Extraction efficiencies of 80–120% are expected for all organic contaminants. If particles remain in the pore waters, filtration through solvent-cleaned glass fibre filters (for example, GF/A Whatman) may be necessary before analyses. Detection limits typically vary from 0.1 to 5 µg/L.

3.5.3 Other pore-water constituents

Ammonia is the most common pore-water toxicant identified by toxicant identification evaluation (TIE) studies (Ho *et al*., 2002; USEPA, 2007c; Ho and Burgess, 2009). The total ammonia concentration in a natural water is the sum of the two chemical species: ionised (NH_4^+) and un-ionised ammonia (NH_3). It is un-ionised ammonia that is primarily responsible for toxicity to aquatic organisms. The proportion of total ammonia that is present as un-ionised ammonia is pH- and temperature-dependent. It is recommended that the pH of sediments be recorded in the field, and the pH of pore water be recorded following extraction. For simplicity, the guideline values are usually expressed as total ammonia (mg NH_3-N/L) and values are provided for different pH values (that is, lower guideline values at higher pH) (ANZECC/ARMCANZ, 2000b; Batley and Simpson, 2009).

Pore waters isolated for ammonia analyses should be stored in containers with minimum headspace, and refrigerated (4°C) if analyses can be made the same day. Otherwise, a drop of sulfuric acid (H_2SO_4) should be added to preserve the samples below pH 2, and they should then be stored cold (4°C) or frozen for later analysis (to be undertaken as soon as feasible). Analyses of ammonia are best undertaken using colorimetic analysis techniques with detection limits of ~10 µg/L. The ammonia-selective electrode method is appropriate when concentrations range between 0.03 and 1400 mg NH_3-N/L.

Cyanide can enter aquatic systems as CN^-, as undissociated/un-ionised hydrocyanic acid (HCN), and as a range of complexes with iron (for example, $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$) or other metals. These complexes have varying stabilities (for example, Zn, Pb, and Cd complexes dissociate in water while those of Ni, Cu and Ag are more stable). Pore waters isolated for cyanide analyses should be stored in containers with minimum headspace, and refrigerated (4°C) if analyses can be undertaken on the same day. Otherwise, sodium hydroxide (NaOH) should be added before refrigeration (4°C). If sulfide is suspected of being present in the isolated waters, lead acetate should be added to remove sulfide before decanting into the final storage. Methods are available for total cyanide, weak acid-dissociable cyanide (free cyanide + acid-dissociable complexes) and free cyanide. Undissociated HCN is the more toxic form, and the guidelines are expressed on this basis (ANZECC/ARMCANZ, 2000b). At pH values between 6.5 and 8.0 (10–30°C water temperature), un-ionised cyanide is >92% of total cyanide, so this calculation and adjustment

of the trigger value is of little practical significance. Pore-water cyanide can be easily analysed using flow injection analysis. The detection limit of this method is 5 µg/L.

Hydrogen sulfide (un-ionised H_2S) is the more toxic form of aqueous sulfide, and the guidelines are expressed on this basis. The determination of hydrogen sulfide is difficult, so the usual analytical procedure involves colorimetric measurement of total sulfide and calculation of the fraction that is present as un-ionised H_2S at the pH. Pore waters isolated for sulfide analyses should be stored in containers with minimum headspace, and refrigerated (4°C) if analyses can be made the same day. Otherwise, zinc acetate and sodium hydroxide (NaOH) should be added (to precipitate the sulfide as ZnS) and then the samples should be refrigerated (4°C). Pore-water sulfide can be analysed easily using the colorimetric method described by Cline (1969). The detection limit of this method is 1–10 µg/L.

Salinity can be an important parameter where there is groundwater intrusion into estuarine or marine sediments. The most reliable method to determine the true or absolute salinity is by complete chemical analysis (Grasshoff *et al*., 1983). However, this is time-consuming and costly and methods based on electrical conductivity should be used instead. Conductivity measurements have the greatest precision.

Dissolved organic carbon (DOC) forms strong complexes with many metals and influences metal bioavailability (Di Toro *et al*., 2001; Besser *et al*., 2003; Strom *et al*., 2011; Campana *et al*., 2013). DOC is generally defined as the fraction of organic carbon that passes through a 0.45 µm filter. Sediment pore waters may have very high DOC concentrations, a considerable portion of which may be colloidal (Chin and Gschwend, 1991; Burdige and Gardner, 1998; Sañudo-Wilhelmy *et al*., 2002). DOC is generally measured using high temperature combustion methods with infrared detection (APHA/AWWA/WEF, 2012).

Pore-water pH may differ from the pH of overlying waters. It should be measured (by standard methods) immediately following isolation, to minimise changes (for example, due to degassing of carbon dioxide, or oxygen intrusion).

3.6 Contaminant bioavailability

Numerous concepts and definitions of bioavailability exist, and methods to estimate it are even more numerous. The concepts and the methods are both important considerations for sediment quality assessments (Chapman *et al*., 1998; Simpson and Batley, 2007; Ahlf *et al*., 2009; Maruya *et al*., 2012; Simpson *et al*., 2013; Besser *et al*., 2014). Bioavailability can be defined as the fractions of contaminants that are available for uptake by an organism of interest, and is therefore organism specific. The bioavailable fraction of the contaminants is directly responsible for observed biological effects. This is related to the chemical behaviour of the contaminant (diffusion, sorption and partitioning) and is closely linked to activity/fugacity, the freely dissolved concentration, and also the uptake kinetics of contaminants associated with ingested solids (dietary exposure). The term bioaccessibility is commonly used in soil science and refers to the accessible quantity of a contaminant that can become available; for example, for biodegradation or accumulation (McLaughlin and Lanno, 2014). Bioaccessibility can be determined with mild extraction schemes (see Sections 3.6.1 and 3.6.4 below).

Although a wide range of chemical methods have been described as assessing 'bioavailability', a more accurate assessment of bioavailability will be achieved using methods that involve exposing the relevant organisms to sediment (in the laboratory and/or the field). However, the use of bioaccumulation in tissues (tissue residues) as a measure of bioavailability may also not reflect the true bioavailable contaminant content in the sediments,

because excretion (of metals) or metabolism of organics will occur to varying extents. The use of organisms to assess bioavailability directly is discussed in Chapter 5.

The consideration of bioavailability becomes very important for sediment quality assessment when guideline values are exceeded and contaminants of potential concern (COPCs) are identified. It is well recognised that total contaminant concentrations are often poor predictors of the risk posed by contaminants in sediments (Simpson and Batley, 2007; Strom *et al*., 2011; Simpson *et al*., 2011; Maruya *et al.*, 2012; Besser *et al*., 2013; Vangheluwe *et al*., 2013). The contaminant exposure, dose to the organism and toxic response observed are all highly dependent on contaminant bioavailability (Simpson and Batley, 2007; Rainbow, 2007; Maruya *et al.*, 2012; Besser *et al*., 2014).

3.6.1 Metal bioavailability

A detailed review of the factors affecting metal bioavailability in sediments has been provided by Simpson and Batley (2007), but new approaches continue to be developed and validated. The prediction of metal bioavailability in sediments is considerably more difficult than in waters because of the greater range of metal-binding phases and processes influencing metal exposure in sediments. The solid-phase speciation (metal binding with particulate sulfide, organic carbon and iron hydroxide phases) and the associated pH and redox conditions (dissolved oxygen penetration) that greatly influence the sediment–water partitioning characteristics (pore-water concentrations and flux rates) and organism behaviour (free living or burrowing, selective feeding) all influence metal bioavailability. Dissolved metals in the pore water, burrow waters or accessed from the overlying water, are generally significantly more bioavailable than those associated with sediment particles. However, the concentrations of contaminants associated with sediments are typically many orders of magnitude higher than those in the dissolved phase. Partition coefficients (K_d = [sediment contaminant, mg/kg]/[dissolved contaminant, mg/L]), are typically of the order 100–10,000). As a consequence, exposure to bioavailable sediment-bound contaminants is an important consideration, as is exposure to contaminated algae or bacteria (for example, via dietary routes). The contaminant speciation in both the dissolved and the particulate phases influences contaminant bioavailability.

The dietary-exposure route may include both living (for example, algae or other benthos) and non-living (for example, organic detritus or sediments) sources of particulate metals. The organism's feeding selectivity and physiology (gut passage time, metal assimilation efficiency from solid) will determine which of these phases contributes more to the exposure. Toxic effects are elicited when a critical dose of a chemical is reached in one or more sensitive compartments of the organism from a combination of dissolved and dietary exposure sources (Luoma and Rainbow, 2005; Rainbow, 2007; Offermann *et al*., 2009; Casado-Martinez *et al*., 2010; Campana *et al*., 2012, 2013).

Metal partitioning between sediment and water, and the speciation within each phase, will differ significantly among the vertically stratified redox zones of sediments. In the oxic surface layer, oxygen reduction is occurring; in the sub-oxic zone beneath that there is nitrate, Mn(IV) and Fe(III) reduction; and in deeper anoxic sediment there is sulfate reduction with sulfide formation and potentially methanogenesis. Thus, while total metal concentrations may be very similar, the bioavailable fraction may differ considerably with sediment depth. However, sediments can be disturbed, for example, by organisms irrigating burrows, plant roots introducing oxygen to depth, or resuspension of surface sediments by currents, and this can modify the dominant metal-binding phases and partitioning of metals between the sediments and pore waters. The potential changes

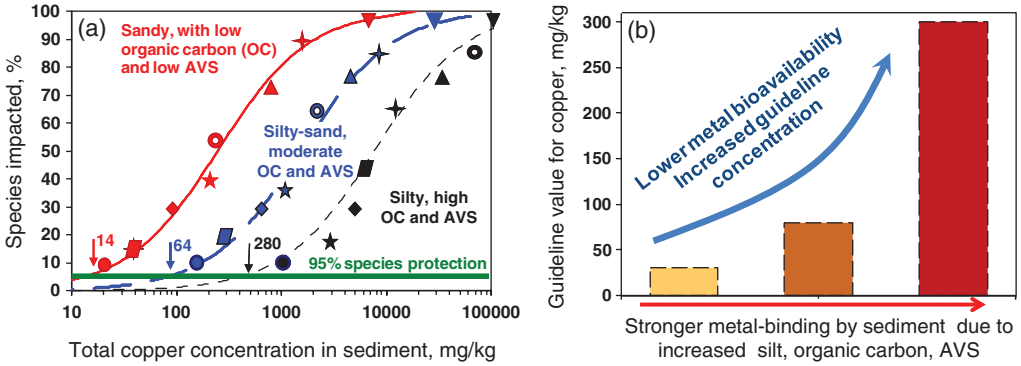

Figure 3.1. (a) Conceptual species sensitivity distributions for toxic effects of copper in sediments with varying properties. Symbols represent different classes of sediment-dwelling biota (for example, algae, amphipods, bivalves, chironomids, copepods, nematodes, snails and worms) (modified from Simpson and Batley, 2007); (b) an example of the influence of sediment properties on the predicted sediment quality guideline value for copper.

in metal partitioning and hence bioavailability when sediment is disturbed may need to be considered for some assessments.

Figure 3.1 illustrates how differences in sediment properties may influence species' sensitivity to contaminants and the predicted effects-thresholds for communities of benthic organisms in sediments. The purpose of the analyses described in this section is to provide information on the major factors that modify the partitioning, bioavailability and risk posed by metal contaminants.

Dilute-acid extractable metals

A significant portion of the total metal concentration (extracted using concentrated acids) may not be bioavailable to organisms (for example, highly mineralised forms). For most metals, the 'maximum bioavailable concentration' can be determined by dilute-acid extractable metal (AEM) analyses. The most common of these analyses involves reacting the wet sediment with cold 1 M hydrochloric acid in a sediment:acid ratio of 1:50 for 1 h. This extraction is analogous to the extraction of metals used in the AVS-SEM (acid volatile sulfide minus simultaneously extracted metals) analysis. It is important to note that this extraction is much stronger than can be achieved by organisms (it is quite a strong acid extraction). Nevertheless, the AEM concentration provides a useful estimate of the 'potentially bioavailable' metal concentration, and it is usually suitable to compare this to the guideline value. Methodological details for SEM determination are provided in Appendix C.

Acid volatile sulfide (AVS)

Submerged sediments frequently accumulate iron and manganese monosulfide (FeS, MnS) and pyrite (FeS_2) phases as a result of the sulfate reduction that occurs naturally through microbial respiration of organic carbon. Owing to the greater availability of sulfate in saline waters, monosulfide concentrations are typically higher in estuarine sediments with ongoing inputs of organic matter (for example, near mangroves) than in freshwater environments. However, submerged acid-sulfate soils may also have considerable monosulfide concentrations if not disturbed by cycles of drying and dessication. Short-term exposure to air or oxygenated water can cause rapid oxidation of a large portion of the monosulfide

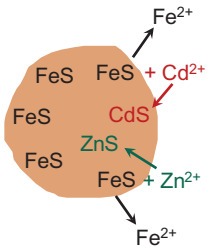

Figure 3.2. Schematic of the exchange of soluble metals in pore waters with AVS (FeS) in sediments.

phases, but prolonged drying is required before pyrite oxidation becomes considerable (Simpson *et al.*, 1998; Rickard and Morse, 2005).

The amorphous monosulfides, FeS and MnS, readily react with several dissolved metals to form metal-sulfide precipitates (Eqn 1, Fig. 3.2). The major sulfide-forming metals include Ag, As, Cd, Co, Cu, Hg, Ni, Pb and Zn (Rickard and Morse, 2005).

$$M^{2+} + FeS(s) \rightleftharpoons MS(s) + Fe^{2+} \quad (1)$$

where M = Ag, Cd, Cu, Ni, Pb, Zn (with Ag and Cu forming Ag_2S and Cu_2S).

The reactive forms of sulfide can be measured as AVS, and two methods are described in detail in Appendix C: both the recommended method, of Allen *et al.* (1991), and the more simple and rapid method of Simpson (2001). As stated there also, it is important to note that, for soils, the methods used for AVS analyses frequently use more concentrated HCl (for example, 6 M HCl), resulting in a different operationally defined sulfide fraction (that is, higher measured concentrations of metal), which is not equivalent to the AVS considered for most sediment quality assessments.

Based on equilibrium partitioning (EqP) theory, for sediments containing a molar excess of AVS over SEM. (ΣCd, Cu, Ni, Pb, Zn), it is predicted that the pore-water concentrations of the SEM will be negligible and should not cause direct toxicity to benthic organisms (Ankley *et al.*, 1996; USEPA, 2005). For chromium, the reduced form, Cr(III), is relatively insoluble and non-toxic compared to Cr(VI). In the presence of AVS, thermodynamics predicts that Cr(VI) will be reduced to Cr(III) and dissolved chromium should not cause direct toxicity to benthic organisms (Berry *et al.*, 2004). Concentrations of AVS are typically much higher in marine than freshwater sediment, but the AVS-SEM model has found applications in both environments (USEPA, 2005; Besser *et al.*, 2013).

In formal guidance for the AVS-SEM approach, the USEPA (2005) refers to equilibrium partitioning sediment benchmarks (ESBs). When SEM-AVS <0, the ESB is not exceeded and sediments should not exhibit toxicity that can be attributed to Ag, Cd, Cu, Pb, Ni and Zn. The ESB metal-mixture procedure of the USEPA (2005) proposes further refinement of the EqP approach to consider partitioning of these metals to both AVS and particulate organic carbon: (SEM – AVS)/f_{OC}, where f_{OC} is the fraction of sediment that is organic carbon. For this latter model, benchmarks of 130 and 3000 μmol/g_{OC} have been proposed, to indicate the risk of adverse effects from these metals as 'low' or 'expected', respectively (USEPA, 2005; Simpson *et al.*, 2012, 2013).

The situations that most significantly limit use of the AVS-SEM approach are the low concentrations and non-permanent nature of AVS in surface sediments owing to the oxidation that occurs when sediment is disturbed by organisms or resuspended by water currents (Peterson *et al.*, 1996; Eriksson Wiklund and Sundelin, 2002; De Lange *et al.*, 2008;

Simpson *et al*., 2012), and the high variability due to seasonal conditions that influence supply of dissolved oxygen, labile organic matter and bacteria-regulated decomposition of organic matter (De Jonge *et al*., 2010). As with all EqP methods, dietary exposure is not explicitly considered.

Metal bioavailability in oxidised sediments

For oxic/sub-oxic sediments where AVS concentrations are low, the bioavailability of metals is strongly influenced by the concentrations of particulate organic carbon (POC), iron and manganese oxyhydroxides, and sediment particle size (Besser *et al*., 2003; Costello *et al*., 2011; Strom *et al*., 2011; Campana *et al*., 2012). Recent studies of such sediments have demonstrated that sediment quality guideline values based on the organic carbon-normalised copper concentration of the <63 μm sediment fraction provide useful predictions of acute and chronic toxicity of copper to benthic marine invertebrates (Strom *et al*., 2011; Campana *et al*., 2012, 2013). For freshwater sediments, the bioavailability of nickel is more strongly influenced by iron than by organic carbon and the most important factors modifying nickel bioavailability and toxicity have been found to be AVS and iron (Costello *et al*., 2011; Besser *et al*., 2013; Vangheluwe *et al*., 2013).

3.6.2 Bioavailability of hydrophobic organic contaminants

Organic contaminants can be divided into hydrophobic (non-polar, water-insoluble) and hydrophilic (polar, soluble) species, and further subdivided as acidic, basic or neutral compounds. The former distinction, based on water solubility, can be related to the compound's octanol:water partition coefficient (K_{OW}). With their lower solubility and greater propensity to bind to sediments, hydrophobic organics tend to accumulate, forming higher concentrations. In practice, hydrophilic organics are typically less persistent than hydrophobic compounds, because they are more amenable to hydrolysis and other-solution degradation processes.

The bioavailability of hydrophobic organic contaminants (HOCs), such as PAHs and total petroleum hydrocarbons (TPHs), and tributyltin (TBT), is strongly influenced by the concentration and form of organic carbon (Di Toro and McGrath, 2000; Koelmans *et al*., 2006; Maruya *et al*., 2012; USEPA, 2003a, 2012). Under the relatively static conditions that exist for many contaminated sediments, a quasi-equilibrium is established between contaminants in organisms, pore waters as a highly bioavailable phase, and solid phases that contain both bioavailable and more inert species (tightly bound or within particles). There is extensive evidence showing that concentrations of HOCs in sediment pore water, particularly of those HOCs that are freely dissolved in pore waters, represent the highly bioavailable fraction and thus are a good indicator of bioavailability (Kraaij *et al*., 2003; You *et al*., 2006; Lu *et al*., 2011; Gschwend *et al*., 2011; Ding *et al*., 2012).

Direct measurement of organic contaminant concentrations in pore waters is difficult because of the challenges involved in extracting sufficient volumes of pore water for analyses, and potential artefacts associated with the extraction methods (see Chapter 2 Section 2.11). Partly due to the difficulties in analysing pore waters, equilibrium partitioning (EqP) models have been a favoured alternative approach for predicting dissolved HOC concentrations in pore waters (see Section 3.6.3 below). A variety of passive sampling methods using both equilibrium mode and kinetic mode (accumulating the organic chemical through time) have been developed to estimate pore-water concentrations of organic contaminants; they are discussed in this section.

Owing to the propensity of HOCs to adsorb to sediment organic carbon and potentially be mobilised in the guts of organisms when ingested, methods have also been developed to target this fraction of bioavailable organic contaminants. The main methods use non-exhaustive extraction; Tenax® extraction and gut fluid mimics are described in Section 3.6.4).

3.6.3 Equilibrium partitioning methods for organic contaminants

Equilibrium partitioning (EqP) models can be used for predicting dissolved concentrations of non-ionic organic chemicals (for example, HOCs) in pore waters (Di Toro *et al*., 1991, 2000; Burgess *et al*., 2013; USEPA, 2013). The EqP approach is based on sediment organic carbon being the major binding phase for HOCs in the sediments. The EqP model holds that HOCs in sediments partition between sediment organic carbon, pore water and benthic organisms. It has been demonstrated that biological responses of benthic organisms to non-ionic organic chemicals in sediments are different across sediments when the concentrations are expressed on a dry weight basis, but similar when expressed on an organic carbon basis (mg/kg OC) (Di Toro *et al*., 2000; USEPA, 2013). Similar responses were also observed across sediments when pore-water concentrations were used to normalise bioavailability (hence the use of passive sampling methods). Detailed guidance on the use of the EqP approach is provided in the EqP sediment benchmark (ESB) procedure documents USEPA (2003b, 2012), which describe procedures for deriving site-specific concentrations for non-ionic organic chemicals in sediment that are protective of freshwater and marine benthic organisms. The ESB approach guides assessment of the additive toxicity of specific classes of toxicants (for example, PAH mixtures and other narcotic organic chemicals).

The ESB approach calculates a final chronic value (FCV) concentration for each specific PAH in sediment (including 18 parent and 16 alkylated PAHs), and for total PAHs as the sum of the quotients of the 34 individual PAHs in a specific sediment divided by the FCV of each individual PAH. Where fewer than 34 PAHs are analysed (for example, alkylated PAHs have not been reported), uncertainly factors are applied to ensure conservative predictions. Limitations of the EqP and ESB approaches include their inability to consider the antagonistic, additive, or synergistic effects of other sediment contaminants in combination with the non-ionic organic chemicals, or the potential for bioaccumulation and trophic transfer of these chemicals.

According to the EqP theory, at equilibrium, if the concentration in any one phase (that is, sediment organic carbon, pore water, or benthic organism) is known, then the concentrations in the others can be predicted:

$$C_d = C_p / (f_{OC} \times K_{OC}),$$

where the dissolved organic chemical concentration in the pore water (C_d, µg/L) is calculated from its particulate sediment concentration (C_p, µg/kg) based on the fraction of organic carbon (f_{OC}) in the same sediment (f_{OC} = %TOC/100) and the organic carbon:water partition coefficient (K_{OC}) for the chemical. Values of K_{OC} can be estimated from the relationship (Karickhoff *et al*., 1989):

$$\log_{10} K_{OC} = 0.00028 + 0.983 \times \log_{10} K_{OW,}$$

where K_{OW} is the octanol:water partition coefficient (Di Toro *et al*., 2000).

The strength of binding of HOCs is often many orders of magnitude greater for black carbon (pyrogenic carbon or soot) than for most other forms of natural organic matter (Accardi-Dey and Gschwend, 2002; Koelmans *et al*., 2006; Maruya *et al*., 2012; USEPA, 2012), and both one- and two-carbon EqP models are available (USEPA, 2012). Coal is both a form of natural organic matter and a form of black carbon to which HOCs are adsorbed

more strongly than to other forms of natural organic matter. The EqP approach is expected to make overprotective predictions when black carbon is not considered. Other available EqP approaches utilise a 'two-carbon' model that estimates the concentrations of chemical in pore water by taking into account the influence of black carbon (USEPA, 2012). The two-carbon model requires information on the speciation of organic carbon (that is, considering both organic carbon and black carbon). However, it should also be noted that natural organic matter and black carbon may exist either as discrete particles or as coatings on inorganic substrates, and not all of the natural organic matter or black carbon may be accessible for binding to HOCs. Particles of coal would appear in sediments as black carbon.

The EqP approach has been used in the derivation of sediment quality guideline values for PAHs (Di Toro and McGrath, 2000), dieldrin and endrin (USEPA, 2003c,d). For sediments having ≥0.2 to ≤10% organic carbon (OC) (dry weight), guideline values normalised to 1% OC have been shown to be valid for a range of sediment types (outside this range the values of 0.2 and 10 are used for the normalisation). This cut-off in organic carbon content (that is, 0.2–10%) is judged to be necessary because at lower organic carbon contents, second-order effects such as particle size and adsorption to non-organic mineral fractions become more important. The normalisation calculation simply involves division of the HOC concentration by the percentage of organic carbon, for cases within the above limits (for example, for a sediment containing 5 mg/kg of total PAHs and 0.55% OC, then the 1% OC normalised concentration is 5/0.55 = 9.1 mg/kg of total PAHs (1% OC); for 15 mg/kg of total PAHs and 3.2% OC, the 1% OC normalised concentration is 15/3.2 = 4.7 mg/kg of total PAHs (1% OC).

Di Toro and McGrath (2000), using EqP-toxicity relationships for PAHs, proposed a final acute value (FAV) and final chronic value (FCV) for total PAHs (sum of the individual PAHs) of 29 and 5.7 μmol/g OC respectively (equivalent to 10 and 50 mg/kg of total PAHs, the Australian and New Zealand SQGV and SQGV-high, respectively (see Chapter 1 and Appendix A), normalised to 1% OC, assuming PAHs have an average molecular weight of 173). While the application of single sediment quality guideline values (SQGVs) to the total PAH concentration neglects the differences in the toxicities of the individual PAHs of the mixture (that is, it treats all mixtures to be similar composition), this SQGV approach generally works successfully given the intended use (that is, if exceeded, it is a trigger for further investigation; see Chapter 1 Section 1.3). In sediments where PAHs are expected to be a major stressor, the EqP-based ESB approach is recommended to provide a more accurate initial assessment of the risk posed by the sum of the individual PAH concentrations. Analogous to this, for the application of other SQGVs, it is recommended that if the ESB guidelines are exceeded, then additional lines of evidence should be considered, so as to make informed management decisions. Passive sampling methods for measuring the freely dissolved concentrations can provide a useful means for validating EqP predictions (see Chapter 2 Section 2.12.2).

3.6.4 Bioavailability methods for organic contaminants

Tenax® extractions (Cornelissen *et al*., 2001; Kraaij *et al*., 2002; You *et al*., 2011) and gut fluid mimics (also referred to as biomimetic methods) (Weston and Mayer, 1998; Turner *et al*., 2001; Voparil and Mayer, 2004) can provide useful information on the potential bioavailability of organic contaminants. These approaches are not substitutes for organisms, but, like pore-water analyses and passive sampling methods, they provide complementary information for assessing the risk of bioaccumulation and toxicity. These methods are often referred to as 'bioaccessibility' methods.

Tenax® desorption technique

Tenax® polyester resin has a strong sorption affinity for a variety of HOCs, and can be used as polymer resin beads or packed into columns with sediments. Tenax® can be used to assess the bioavailable portion of HOCs, and relies on the principle that HOCs of different bioavailability will display distinct desorption rates from sediment particles (Cornelissen *et al.*, 1997; You *et al.*, 2011). Those HOCs with faster desorption kinetics are considered to be more bioavailable (Cornelissen *et al.*, 2001; ten Hulscher *et al.*, 2003; You *et al.*, 2006; Landrum *et al.*, 2007; Mackenbach *et al.*, 2012).

In the application of Tenax® extractions (MacRae and Hall, 1998; You *et al.*, 2011), the Tenax® beads are added to sediment slurries in glass containers and the containers are then continuously rotated. At pre-determined times, the Tenax® beads are separated from the sediment by centrifugation (Tenax® beads float in water); the target HOCs are desorbed from the Tenax® beads using solvent extraction and then analysed using standard methods (additional solvent clean-up steps may be required). Consecutive Tenax® extractions may be undertaken with fresh Tenax® added to resume the desorption process. Landrum *et al.* (2007) showed that 6-h Tenax®-extractable HOC concentrations successfully predicted the accumulation of HOCs in organisms exposed to field-contaminated sediments. As an alternative to Tenax®, XAD-2 (a polystyrene copolymer) has also been applied as an adsorbent phase (Lei *et al.*, 2004; Simpson *et al.*, 2006). The capacity for Tenax® beads to adsorb HOCs is very high (described as an 'infinite' sink), and they have the potential to remove the entire pool of HOCs that have desorbed from a sediment during a given timeframe. Compared to techniques such as SPMEs, Tenax® requires only a single time-point treatment (for example, 6 h or 24 h), making it very effective for HOCs with short environmental half-lives or for low concentrations. However, the methods are not applicable for *in situ* applications (Cui *et al.*, 2013).

Gut-fluid mimic techniques

Deposit-feeding and some suspension-feeding organisms accumulate many heavy metals and HOCs via the ingestion of sediment (Lee *et al.*, 2000; Weston *et al.*, 2000; Voparil *et al.*, 2004; Tan *et al.*, 2013). However, a proportion of most contaminants is not desorbed from the particles while in the gut, and passes out of the organism via the faeces (Wang and Fisher, 1999). The bioavailability of particle-associated contaminants to deposit feeders can be estimated using digestive fluid extracted from organisms (Tan *et al.*, 2013), or digestive fluid mimics such as the protein bovine serum albumin (BSA) and surfactants (for example, sodium taurocholate), targeting metals or organic contaminants (Mayer *et al.*, 1996; Chen and Mayer, 1999; Ahrens *et al.*, 2001; Weston and Maruya, 2002; Voparil and Mayer, 2004) or, for metals, by using model enzymes (pepsin or trypsin) (Turner and Olsen, 2000; Turner *et al.*, 2001; Turner, 2006; Turner and Radford, 2010).

In these methods, sediments are incubated *in vitro* with the digestive fluid (or with the chemical surrogate) and the amount of the particle-associated contaminant that is desorbed in the fluid is then quantified on the presumption that sediment-associated contaminants must first be solubilised in order to be bioavailable. While these methods do not enable the amount of chemical accumulated by an organism to be predicted, they are useful for qualitatively assessing the relative bioavailability of certain chemicals, and more closely mimic the chemical conditions of the digestive environment of deposit-feeding organisms than do conventional chemical digests like dilute acids. Because subsequent absorption of the solubilised contaminant across the gut wall is not addressed, the solubilised contaminant may still not be bioavailable, but the method gives an upper limit for the

concentration of contaminant that is likely to be made bioavailable from a given sediment during gut passage (Weston and Mayer, 1998). Regression analyses of gut-fluid-extracted contaminant concentrations versus bioaccumulated body burdens revealed strong positive correlations for several contaminants, suggesting that gut-fluid extractions might be considered as predictors of bioaccumulation (Lawrence *et al.*, 1999; Weston and Maruya, 2002). Tan *et al.* (2013) found that concentrations of metals in the coelomic fluid of a sipunculid (or peanut) worm provided useful information on metal bioavailability in marine sediments.

3.7 Nutrients

The major nutrient elements of environmental concern in sediments are nitrogen and phosphorus. Both are present in organic and inorganic forms. Inorganic forms of nitrogen include nitrate, nitrite and ammonia. Organic nitrogen undergoes bacterial degradation and denitrification via ammonia, nitrite, and nitrate, ultimately to elemental nitrogen, N_2 (Jørgensen and Revsbech, 1989). In oxygen-limited systems, these reactions can stop at ammonia. Phosphorus exists as phosphates, both monomeric and polymeric, and in sediments is usually bound with iron (Harris, 2001). Considerable phosphorus and nitrogen can also be incorporated by bacteria and benthic microalgae and it is important to consider living microscopic benthos as part of the sediment structure.

3.7.1 Sample storage and preparation

Sediments for nutrient analysis can be stored frozen (–20°C) indefinitely in a polyethylene container that has been prewashed with phosphorus-free detergent. Total nutrient analyses are best undertaken on an aliquot of sediment that has been homogenised when moist, then freeze-dried and ground before analysis.

Phosphorus

Phosphorus is extracted from sediments using microwave-assisted digestion with nitric acid alone or a mixture of nitric and sulfuric acids (Maher *et al.*, 2001). Phosphorus solubilised by nitric acid can be measured by ICPMS, while phosphorus solubilised by combined nitric and sulfuric acids is measured colorimetrically (Esslemont *et al.*, 2000).

Nitrogen

Total nitrogen is commonly measured by two methods. The simplest procedure uses a carbon, hydrogen and nitrogen (CHN) analyser, for which the sediment only needs to be finely ground. However, the CHN analyser typically requires only 10–100 mg of material, which means the values obtained may not be fully representative of the sample (Phillips *et al.*, 2011). Alternatively, total N can be measured in larger subsamples of sediment, typically 0.5 g, digested with a potassium peroxodisulfate-sulfuric acid mixture (Maher *et al.*, 2002).

Ammonia

In sediments, ammonia is only found in pore waters. It is regularly measured, because of its toxicity to organisms. Pore-water samples for ammonia can be stored frozen (–20°C), in either glass or polyethylene containers away from possible sources of contamination such as fish and other biota, and should be analysed within two weeks. Ammonia is measured colorimetrically (see Section 3.5.3 above, and APHA/AWWA/WEF, 2012).

3.8 Measuring contaminant fluxes

The measurement of fluxes of contaminants from sediments to overlying waters is useful for understanding the influence of sediments on overlying water quality. Generally, sediments that exhibit a high flux of contaminants to the water column will represent a greater risk to the environment than those with lower fluxes. In sediments that have similar concentrations of a contaminant, greater fluxes of the contaminant may also indicate greater bioavailability of that contaminant and a greater risk of toxicity to benthic biota. Flux measurements can also provide useful information to assist with assessment of dominant exposure pathways.

Normally the release of contaminants from sediments will be driven by partitioning from sediments to pore waters and then a concentration gradient from pore waters to the overlying water. If concentrations of contaminants in pore water are higher than those in the overlying water, there will be a net positive diffusional flux of contaminants to the overlying water. A flux is defined as the movement of a contaminant (mass) through a given surface area per unit of time, and it can be calculated using Fick's first law of diffusion (Boudreau, 1997; Di Toro, 2001): The concentrations of contaminants in the pore waters of surface sediments (0–1 mm depth) will often control the flux of contaminants to the overlying waters.

The flux is given by:

$$F = -Ds \times \delta c/\delta z,$$

where F is the flux, Ds is the diffusion coefficient of the contaminant in the sediment, and the partial differential is the concentration gradient at the sediment–water interface. The sediment diffusion coefficient can be estimated from the porosity of the sediment surface layer, ϕ, and the diffusion coefficient of the contaminant in water (D): $Ds = \phi D$.

Releases of contaminants from particles will also occur as a result of changes in redox potential and pH (Boudreau, 1997). Metals, in particular iron and manganese, are sensitive to redox potential changes, being oxidised from the reduced forms Fe(II) and Mn(II) to Fe(III) and Mn(IV) (MnO_2) (for example, as the dissolved reduced forms diffuse into oxygenated surface sediments; or through organisms introducing oxygenated waters into deeper sediments) (for example see Chapter 2 Fig. 2.1). These and other metal or metalloid (for example, As(III/V), Se(IV/VI), and Cr(III/VI)) transformation processes may be driven by microbial processes or benthic algal metabolism. These oxidation processes also tend to release H^+, so there can be a net decrease in pore-water pH that will enhance the possibility of metal dissolution from particulates.

Changes in pH associated with the above reactions can alter the partitioning of some organic contaminants, while microbial and chemical transformation can also result in transformation and degradation processes for other organics. In the case of selenium, for example, inorganic selenium undergoes biomethylation to volatile species such as dimethylselenium, $((CH_3)_2)Se$ (Chau *et al*., 1976; Neumann *et al*., 2003). Such biologically mediated fluxes are dependent on water temperature; thus sampling programs will need to have a temporal component that includes at least winter and summer sampling. As benthic algal production and respiration are dependent on light, there might be a need to consider fluxes under both light and dark conditions if this is deemed important.

However, it is important to recognise that, under certain conditions, the direct release of pore water from sediment to overlying water can occur due to physical disturbances such as those caused by bioturbation facilitated by burrowing organisms (Aller *et al*., 2001; Atkinson *et al*., 2007). Numerous field studies have provided evidence for the importance of sediment resuspension for hydrophobic organic contaminant transport and internal

recycling in aquatic ecosystems (Latimer *et al*., 1999; Bogdan *et al*., 2002). Laboratory experiments have shown significant increases in the concentrations of hydrophobic contaminants in the overlying water after simulated resuspension events (Feng *et al*., 2007; Schneider *et al*., 2007), typically correlated with the increased concentration of suspended particles. Resuspended sediment particles are exposed to a new chemical environment, which may promote the desorption and release of associated contaminants into the water phase as truly dissolved chemicals, which is generally the most bioavailable fraction of these chemicals to pelagic organisms.

There are numerous ways that fluxes can be measured. These include the use of:

- passive sampler measurements that provide a concentration profile (see Chapter 2 Sections 2.11 and 2.12);
- benthic chambers (domes) (in the field) (see below); or
- corer–reactors (in the laboratory) (see below).

Concentrations of metals in pore water can be determined using sediment peepers (dialysis samplers), and DET and DGT 'diffusive equilibrium' and 'diffusive gradient' techniques in thin films (see Chapter 2 Sections 2.11.2 and 2.12.1). Peepers equilibrate pore-water solutes in chambers at a resolution of 0.5–2 cm, while DET equilibrates solutes in a thin hydrogel at resolutions of 0.3–2 mm. DGT samplers accumulate solutes on a binding layer which can be measured at resolutions from 0.1 to 1 mm. When these samplers are deployed both above and below the sediment–water interface, the measured concentration gradient at the interface can be used to estimate a flux, assuming the transfer is purely by diffusion.

3.8.1 Flux measurements using domes

Domes are hemispherical Perspex® benthic chambers (Fig. 3.3) that can be positioned in contact with the sediments and left in place for a fixed sampling period. They are used extensively to measure nutrient fluxes (Berelson *et al*., 1998) and have recently been applied to the measurement of dimethylselenium (Schneider, 2015). Commercially available domes typically have a diameter of 295 mm and a height of 250 mm. Each dome has an inlet and an outlet connection point on the top. Small water samples can be drawn out through these ports. For larger volume samples, a length of Teflon® tubing (for example, 12 mm outer diameter, 10 mm internal diameter) can be connected. A 12 m length of tubing is sufficient to collect ~1 L. Attached to the Teflon® tube is an in-line pump to circulate the water within the dome and the associated tubing in order to balance the concentrations within the system.

Water can be collected by detaching the Teflon® tube from the dome and transferring its contents to a 1 L bottle (the choice of bottle is defined by the substance being sampled). The ends of the tube are closed using gloved hands, to avoid water loss and exchange when carrying the tube to the Teflon® bottle. A dissolved oxygen (DO) meter can be attached to the top of the chamber to continuously measure oxygen concentrations in the water inside the chamber. In order to reflect natural conditions, it will be important that the water inside the domes does not go anoxic.

Domes can be used to measure fluxes of nutrients, ammonia, metals, volatile organometals (for example, dimethylselenium) and organic contaminants.

3.8.2 Flux measurements using corer–reactors

A convenient way to study contaminant fluxes in the laboratory is to use corer–reactors (Jung *et al*., 2003). These consist of ~16 cm diameter × 47 cm long Perspex® (acrylic) tubes

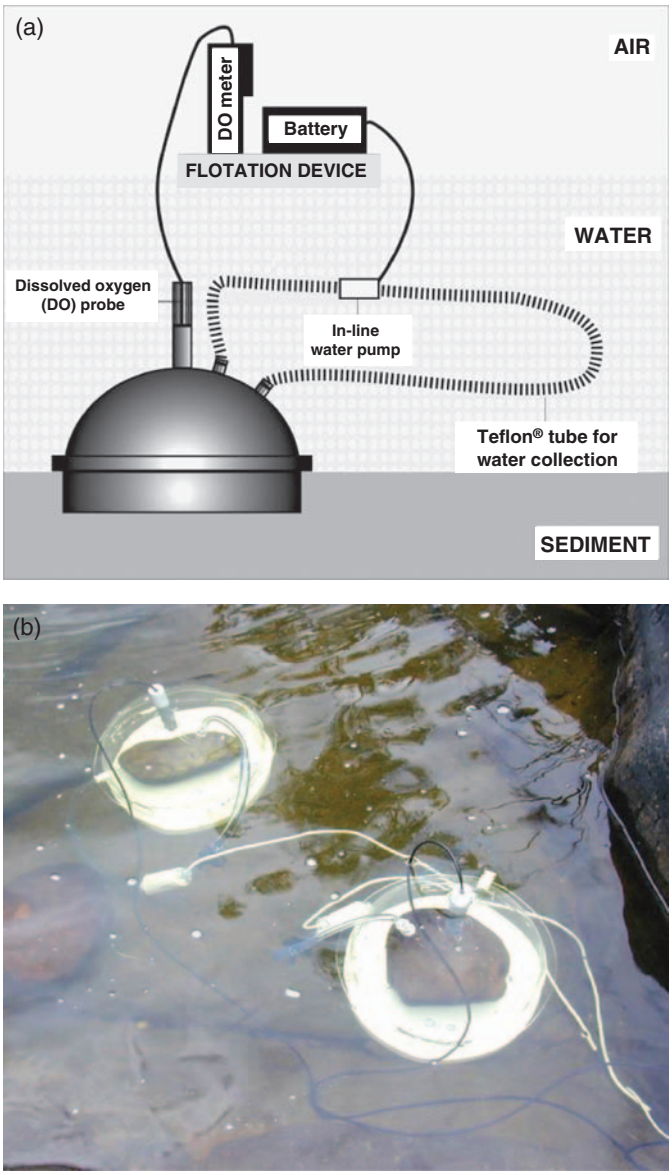

Figure 3.3. (a) Schematic diagram and (b) photograph of field-deployed sediment domes. An in-line pump circulates water within the dome to balance the concentrations of contaminants within the system (from Schneider, 2015). DO = dissolved oxygen.

that can be used to collect an intact sediment core, to a depth of around 20 cm (Fig. 3.4). Once the sample has been collected an acrylic base is screwed onto the bottom of the corer; the remainder of the corer is filled with water and an acrylic screw cap is fitted to the top. The cap contains a stirrer, a sampling port and ports for a DO probe and, if required, an air bubbler. The unique combination of sampler and reactor means that it is possible to overlay a relatively undisturbed core with water that is gently stirred and, if required, aerated. Regular sampling of the overlying water for analysis of released contaminants yields data that can be converted to a flux. Because the overlying water is not replaced, concentrations

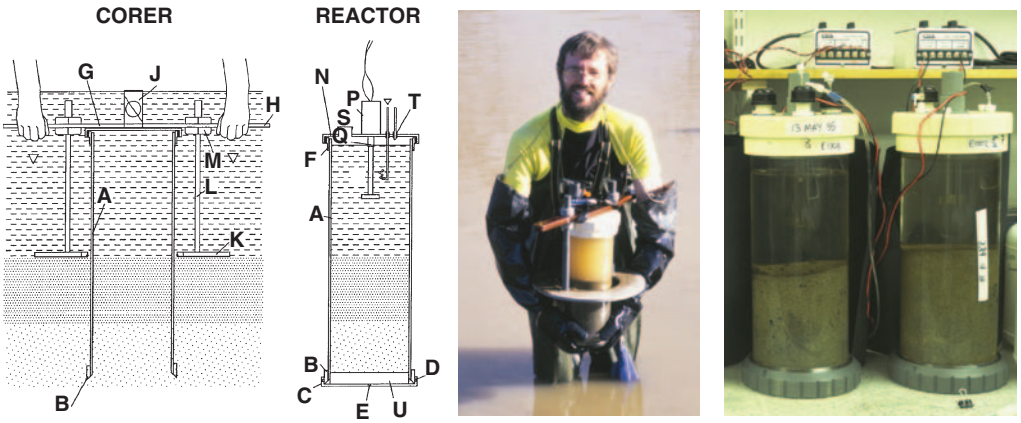

Figure 3.4. Design of the corer–reactor in both corer (left) and reactor (right) modes (a). (A) Acrylic corer tubing; (B) annular acrylic reinforcing section; (C) polyvinyl chloride (PVC) base-sealing cap; (D) nitrile O-ring; (E) hole to let air and water escape; (F) acrylic top collar; (G) PVC corer lid; (H) wooden handle; (J) PVC ball valve; (K) annular PVC stopping plate; (L) PVC rods; (M) PVC nuts; (N) reactor lid; (P) direct current motor; (Q) acetal coupling collar; (R) stirrer blade; (S) porthole for probes and sampling; (T) Luer-lock fittings; (U) ethylvinyl acetate (EVA) foam core plug. (From Jung *et al.*, 2003 with permission). Photographs (b, c) show it in use in the field and laboratory.

will eventually reach equilibrium with the pore-water concentrations. Measurements are typically undertaken for no more than 2 days.

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4

Sediment ecotoxicology

Stuart L. Simpson and Anu Kumar

4.1 Introduction

Contaminants in sediments may be directly toxic to aquatic life or can be a source of chemicals for bioaccumulation in the food chain. This chapter deals with the toxicity testing of sediments using laboratory-based procedures in which live organisms are exposed to field-collected whole sediments and pore waters. Toxicity tests measure the cumulative effects of all bioavailable contaminants and their interactions as mixtures. The toxicity test procedure used can have a major influence on the outcome of the assessment and no one organism or test is best suited for all sediments. Different benthic species have different sensitivities to different toxicants, and a range of organisms having differing exposure pathways (feeding strategies and behaviours) and test endpoints (for example, survival, development, growth, reproduction) should be used for ecotoxicological assessment of contaminated sediments. Sediment toxicity tests can be used to:

- determine the relationship between contaminant (toxicant) concentrations, exposure, bioavailability and toxicity;
- investigate interactions between toxicants and other stressors, such as elevated nutrient concentrations;
- compare the sensitivities of different organisms;
- determine the spatial and temporal distribution of potentially toxic sediments;
- measure toxicity as part of product licensing, compliance or safety testing;
- determine effects-thresholds or site-specific management limits for sediment contaminants;
- rank areas for clean-up (including by dredging); and
- estimate the effectiveness of remediation or management practices.

To achieve a sound ecotoxicological assessment, there are many principles that should be considered. These range from essential aspects of experimental design to demonstrate that the desired exposure has been achieved, through to appropriate control and reference sediments and endpoints and ultimately the achievement of an unbiased analysis of the results. While different principles will apply, depending on the assessment needs, it is useful to consider the following list, provided by Harris *et al.* (2014) and slightly modified here to align more closely with sediment quality assessment. Explanations are in parentheses.

(i) Ensure adequate planning and good design.
(ii) Define the baseline (to define what is a normal response for unexposed organisms in a range of sediment types).
(iii) Include appropriate control sediments and reference sediments (with sufficient samples, detailed characterisation to ensure their properties match the test sediment, and reporting of the properties).
(iv) Use appropriate exposure routes (both dissolved and dietary, for sediments) and concentrations (relevant to the environment being assessed).
(v) Define the exposure (measure actual concentrations; and estimate the potential bioavailable fraction, in the various water–sediment phases present during the tests).
(vi) Understand your tools (gather knowledge about the particular test organism; for example, density/cannibalism issues).
(vii) Think about statistical analysis of the results when designing an experiment (consider exposure concentrations and numbers of organisms, because these determine the statistical power of the results).

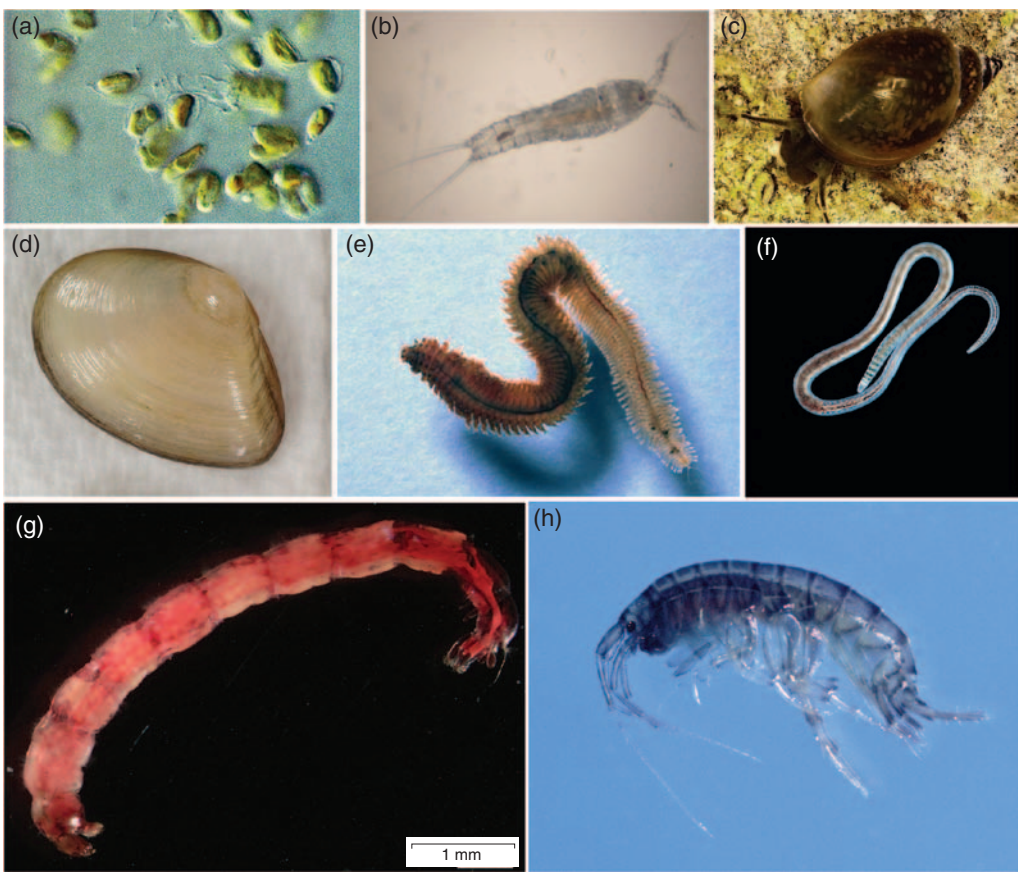

Figure 4.1. Common types of organisms used in whole-sediment toxicity tests: (a) benthic algae (typical size range for test species 5–20 μm), (b) copepod (0.5–3 mm), (c) snail (5–20 mm), (d) bivalve (5–30 mm), (e) polychaete (40–100 mm), (f) oligochaete (40–100 mm), (g) chironomid (3–10 mm) and (h) amphipod (3–20 mm) (see Fig. 1.2 in Chapter 1 for a conceptual model of potential exposure routes in sediments).

(viii) Consider the concentration–response (so that 'unusual' patterns of response can be easily identified for further analysis).
(ix) Repeat the experiment (particularly where results are unexpected and/or borderline).
(x) Consider confounding factors (such as temperature, salinity, disease, and exposure to multiple unmeasured substances/stressors).
(xi) Consider the weight of evidence (is the exposure–dose–response plausible, and do field and laboratory results agree?).
(xii) Report findings in an unbiased manner (address uncertainties; do not over-extrapolate).

Although many of these twelve considerations are not discussed individually below, all are essential for ecotoxicological assessments of sediments, together with appropriate quality assurance and control (QA/QC).

This chapter aims to provide a broad overview of sediment ecotoxicology considerations, covering the selection of appropriate organisms (see Fig. 4.1) and test procedures and data analysis, and the extension of methods for toxicity identification evaluation (TIE) and *in situ* testing. The chapter focuses on testing to identify toxicants in bottom sediments and associated pore waters rather than in overlying waters and associated suspended sediments. For bottom sediments, to capture all exposure pathways, it is preferable to use whole-sediment toxicity tests with sediment-dwelling organisms or biota that live in close association with the sediments, rather than toxicity tests on pore waters isolated from sediments. Appendices D–I describe whole-sediment toxicity test methods using specific species of benthic algae, amphipods, copepods, polychaetes, oligochaetes, bivalves, chironomids and snails.

4.2 Contaminant exposure pathways

Most sediment-dwelling organisms are exposed to contaminants by direct or inadvertent ingestion of sediments and by uptake from pore water and overlying water (Griscom and Fisher, 2004; Rainbow, 2007; Simpson and Batley, 2007; Campana *et al.*, 2012; Proulx and Hare, 2014). Contaminants are released from sediment to the water column both by the diffusive flux of dissolved chemicals in the pore waters (Di Toro, 2001, Erickson *et al.*, 2005), and through physical disturbance (such as sediment resuspension by wave and current actions and dredging and trawling) and biological actions (Aller *et al.*, 2001). The dissolved fluxes are a source of dissolved contaminants to pelagic and benthic organisms, while the resuspended particles are a source of particulate contaminants to suspension-feeding organisms. Regular resuspension of sediments also maintains contaminants in oxidised forms within surface layers. For many benthic organisms, the contaminant exposure at the sediment–water interface is the most relevant for assessments because feeding occurs on organic-rich particulates from this region (many organisms live in and feed on surface sediments; bivalve siphons often do not protrude far into the overlying water). Field studies have provided evidence of the importance of sediment resuspension for transport and internal recycling of hydrophobic organic contaminants in aquatic ecosystems (Latimer *et al.*, 1999; Bogdan *et al.*, 2002). Laboratory experiments have shown a significant increase in the concentration of hydrophobic contaminants in the overlying water after simulated resuspension events (Feng *et al.*, 2007; Schneider *et al.*, 2007), typically correlated with the increased concentration of suspended particles.

Deposit feeders that ingest organic and inorganic particles, obtained from either the sediment surface or within the sediments, are among the organisms which play the most important role in sediment bioturbation. Although some species of benthic fish including European carp are known to influence resuspension of sediments, the process of bioturbation itself is particularly determined by burrowing activities of infaunal invertebrates – that is,

those that live within the surface sediment. These organisms can exert their effect on the sediment itself and on sediment–water interface exchanges in various ways, depending on sediment reworking mechanisms, and on the size, abundance and density of species. Rapid mixing of sediment particles and contaminants within the sediment is one aspect of bioturbation described for polychaete and oligochaete worms. These worms ingest sub-surface bottom sediments and convey them to the sediment–water interface as faecal pellets. A second interesting aspect of bioturbation is the ability of various sub-surface deposit feeders to increase the sediment–water surface area by their feeding and continuous burrowing activity, and to increase fluxes of oxygen, nutrients and toxicants in pore water and at the sediment–water interface. Significant increases in the movement of sediment-associated contaminants from sediment to pore water and overlying water are described by various authors (Green and Chandler, 1994; Briggs *et al.*, 2003; Hedman *et al.*, 2009; Josefsson *et al.*, 2010). In addition, biologically mediated particle resuspension can be substantial and even exceed resuspension caused by physical disturbances in some environments. It has been demonstrated that fluxes of methyl mercury from sediments in Boston Harbor, USA, are correlated with the density of infaunal burrows, which suggests that this is likely to be an important process of contaminant remobilisation in some areas (Benoit *et al.*, 2009).

4.3 Selection of organisms for toxicity tests

The test organism used will have a major influence on the outcome of the tests. No one organism is best suited for all sediment types and assessment scenarios. Generally, a range of organisms with differing behaviours and exposure pathways should be used for ecotoxicological assessment of contaminated sediments.

As general guidance on the selection of organisms for biological tests with sediments (ASTM, 2008a,b, 2010, 2014), ideally, the test organism should:

- be of a type that has direct contact with sediment during normal behaviour;
- have a demonstrated sensitivity to a range of contaminants of interest found in sediments;
- be tolerant of a broad range of sediment physico-chemical characteristics (for example, grain size);
- be compatible with selected exposure methods and endpoints;
- be suited to standardised application and quality assurance procedures, thereby allowing inter-laboratory comparisons;
- be readily available from culture or through field-collection;
- be easily maintained in the laboratory;
- be easily identified (especially when collected from the field);
- have short to moderate (days to weeks) life-cycles to enable reproduction to be assessed without the need for long (months) test durations;
- be ecologically or economically important;
- have a broad geographical distribution, be indigenous (either at present or in the past) to the site being evaluated, or have a niche similar to indigenous organisms of concern (for example, be in a similar feeding guild or have behaviour similar to the indigenous organisms); and
- have its response confirmed with responses of natural populations of benthic organisms.

Few test species in use worldwide meet all of these criteria, but these criteria should be considered carefully when planning sediment quality assessments. It is very important that there be a proven sensitivity of the test species to the contaminants of concern, and

knowledge of the route of exposure, because exposure pathways are readily affected by sample handling techniques.

4.3.1 Pore-water tests

For the purpose of assessing potential effects of contaminated sediments, ecotoxicological tests on pore waters isolated from sediments are not as desirable as whole-sediment tests (Chapman *et al.*, 2002; Anderson *et al.*, 2004; Word *et al.*, 2005). There are particular concerns associated with pore-water tests; for example:

- many benthic species ingest sediments, but that dietary route of exposure to contaminants is not measured in pore-water tests;
- sediment-dwelling organisms (if used) may be stressed by the absence of sediment;
- the volumes of pore water required for toxicity testing can be difficult to acquire; and
- once isolated from sediments, pore waters are generally unstable and their chemistry (including contaminant concentrations) can change rapidly. Of particular concern are the potential for loss of contaminants through adsorption to containers, and the potential for contaminants or water constituents to oxidise which can lead to acidification (for example, Fe(II)) or volatilisation (and a need for the pore waters to be replaced by newly isolated pore-water samples, regularly during tests).

Sediment pore-water tests for estuarine and marine waters typically employ microbial (bacteria) and algal species, or small or juvenile invertebrates or pelagic embryo-larvae stages of organisms such as sea urchins, bivalves and copepods, and for marine and freshwaters they use cladocerans (water fleas) and fish embryo-larval stages (Carr and Nipper, 2003; Anderson *et al.*, 2004).

Sediment elutriates are not considered an appropriate surrogate for pore waters in pore-water toxicity tests, but they are useful for assessing the release of contaminants from dredged sediments as part of the assessment of sediments for seawater or freshwater disposal (NAGD, 2009). Methods for the preparation of sediment elutriates are described in Chapter 2 Section 2.13 and by ASTM (2008a).

Solvent extracts of sediments are not recommended for toxicity testing, but may assist in identifying which of the organic chemicals within a mixture are contributing to toxicity observed in pore-water and whole-sediment tests (for example, as part of effect-directed analysis schemes). For some bioanalytical methods (for example, cell-based bioassays; see Section 4.9 below), solvent extracts are used to extract a 'maximum bioavailable' concentration of organic contaminants for use in these assays. Partial extraction techniques (for example, using Tenax®; see Chapter 3 Section 3.5.4), and passive sampling methods (for example, solid phase microextraction (SPME); see Section 2.12.2) are used to provide closer estimates of the fractions of chemicals present in pore waters (Li *et al.*, 2013).

4.4 Toxicity test species

The sensitivities of benthic organisms to contaminants will differ between species and contaminants because of differences in organism behaviour (burrowing, feeding, life-cycles), and contaminant exposure pathways (for filter or deposit feeders), and the properties of the contaminant (such as partitioning and bioavailability). It is therefore important in sediment quality assessments to use a suite of tests and organisms that have different feeding strategies and behaviours, to cover all potential routes of exposure. For whole-sediment tests, these may include bacteria, benthic algae living at the sediment–water interface (water route), amphipods (free-living or tube-dwelling), bivalve clams, insect larvae such as chironomids

(midges) and mayflies, meiofuana such as harpacticoid copepods and nematodes, worms (oligochaetes in freshwater, polychaetes in marine water) and snails. Large crustaceans such as crabs have not yet been used extensively for whole-sediment tests despite their close association with sediments and many species being very well studied with respect to effects of contaminants in water-only exposures (Rodrigues and Pardal, 2014). Few whole-sediment tests have been developed using plants, but a standardised sediment contact test has been developed using the freshwater macrophyte *Myriophyllum aquaticum* (Feiler *et al*., 2014). For pore-water and elutriate tests, microalgae (freshwater or marine), macroalgae (marine), small invertebrates or larval/juvenile development tests are recommended (for example, with sea urchin or scallop). An ASTM guide discusses many of the important considerations for the selection of resident species as test organisms (ASTM, 2012). Brief descriptions of organisms commonly used for sediment toxicity testing are provided below.

A range of aquatic species frequently used for standardised whole-sediment tests is shown in Tables 4.1 and 4.2. Additional overviews and discussions of a wide range of laboratory toxicity test methods have been provided over the past 10 years, varying in their details and forms of information (Burton *et al*., 2003; Anderson *et al*., 2004; Ireland and Ho, 2005; PIANC, 2006, ASTM, 2010a, 2014; Besser *et al*., 2013). Detailed methods for Australian species are provided in Appendices D–I:

(i) for marine sediments, the benthic alga *Entomoneis* cf *punctulata* (Appendix D), the amphipod *Melita plumulosa* (Appendix E), the harpacticoid copepod *Nitocra spinipes* (Appendix F), and the bivalve *Tellina deltoidalis* (Appendix G); and
(ii) for freshwater sediments, the chironomid *Chironomus tepperi* (Appendix H), and snail *Physa acuta* (Appendix I).

4.4.1 Bacteria

Bacteria are an important component of all sediments, influencing the degradation of organic matter, the cycling of nutrients, and acting as a food source for many benthic organisms. Contaminants may disrupt the metabolic processes of bacteria and thus influence key sediment functions and bacteria populations (Environment Canada, 2002). The bacterial toxicity test Microtox® uses the marine luminescent bacterium *Vibrio fischeri* and is a common test for waters and, to a lesser extent, sediment. *Vibrio fischeri* divert ~10% of their metabolic energy to a metabolic pathway that converts chemical energy, through the electron transport system, into visible light, a pathway intrinsically linked to the respiration of the cell. Hence, a change in cellular metabolism or a disruption of the cellular structure in *V. fischeri* results in a change in respiration and a concomitant change in bioluminescence (Ross, 1993). The Microtox® assay determines the decrease in light output of *V. fischeri* after they have been exposed to sediments for 20 minutes, compared to unexposed controls. Although this acute toxicity test is rapid and commercially available as a test kit with standard testing protocols for solid-phase samples (Azur Environmental, 1998), some important limitations should be considered. After exposure of *V. fischeri*, each replicate test solution is filtered to remove sediment particles that could interfere with the measurement of the luminescence. However, this process also removes sediment-bound bacteria, which results in false-positive results. This problem is particularly bad for fine (<63 μm) sediment with a large surface area for bacteria to bind to. When testing field sediments, these uncertainties can be assessed by use of a sediment control with matching grain size, or a series of sediment controls. In addition, the Microtox® method refers to using pore water, elutriates and solvent extracts as indicators for sediment toxicity, but, as discussed above, there are

significant limitations in using these sediment fractions for pore-water tests (Section 4.3.1). Sediments from freshwater environments can also be tested with saline water but the environmental relevance of this procedure is questionable.

In summary, the Microtox® test has become well established and, due to the simplicity and rapid nature of the test, it is commonly used worldwide and is a useful tool for screening and quickly ranking large numbers of sediments to identify areas of concern. However, it should never be used alone for decision-making because of the likelihood of false-positive results. While standard methods for Microtox® have not been published, the method is well described in peer-reviewed literature (for example, Environment Canada, 2002; Doe *et al*., 2005).

4.4.2 Algae

Benthic algae (microphytobenthos) play an important role in stabilising sediments in shallow water environments, modulating chemical transformations and remobilising metals at the sediment–water interface, and providing habitat. They are a common food source for many invertebrates and hence have the potential to facilitate transfer of sediment contaminants to higher trophic levels. Even though benthic algae do not ingest sediment particles, they are in intimate contact with sediments and associated pore waters, the latter representing the exposure pathway. Difficulties in accurately quantifying microalgae in sediments have hampered growth-rate inhibition bioassays in sediment with a >10% fine fraction (Moreno-Garrido *et al*., 2003). Algal photosynthesis and enzyme inhibition have been found to be more suitable than inhibition of algal growth, as endpoints for algal whole-sediment tests, because toxic effects are not masked by stimulation from ammonia in sediments (Munawar and Munawar, 1987; Blaise and Ménard, 1998; Adams and Stauber, 2004). Flow cytometry, a rapid method for measuring the light scattering and fluorescence properties of cells in a moving fluid, is very suitable for these tests (Adams and Stauber, 2004). The flow cytometric toxicity test requires considerable investment in equipment and skilled operators, but it is relatively easy to train operators in its routine use once the individual test protocol is established. Appendix D provides instructions for undertaking whole-sediment ecotoxicology tests using the benthic marine algae *Entomoneis* cf *punctulata*.

4.4.3 Amphipods and mysids

Amphipods are an abundant and important component of most freshwater (rivers and streams), estuarine and marine benthic communities. They are important in leaf-litter processing and as a principal prey item of many birds, fish and larger invertebrates, and hence they are a key conduit for the trophic transfer of contaminants from sediments to higher trophic levels. Amphipods are ecologically important, and their wide distribution and high abundance, their ease of handling and suitability for culture in the laboratory or field-collection, together with their sensitivity to contaminated sediments, make them appropriate species for sediment toxicity testing. Many amphipod species directly ingest sediment particles and are therefore directly exposed to sediment-bound contaminants along with contaminants in pore waters and overlying waters. Amphipods meet many of the criteria used for selecting test organisms for whole-sediment toxicity tests, and a range of standardised or peer-reviewed methods exist for assessing survival, reproduction, growth and sediment avoidance (Castro *et al*., 2006; Scarlett *et al*., 2007; Greenstein *et al*., 2008; Simpson and Spadaro, 2011; ASTM, 2014). Commonly used amphipod species include *Eohaustorius estuarius*, *Leptocheirus plumulosus*, *Rhepoxynius abronius* (estuarine–marine) and *Hyalella azteca* (to 15‰ salinity) (North American species), *Corophium volutator*, *Gammarus locusta*, *Corophium multisetosum* (European species) and *Melita plumulosa*

(Australia) for estuarine–marine sediment tests, and *Hyalella azteca* (North America) and *Gammarus pulex* (Europe) for freshwater sediment tests. Amphipods have relatively short life-cycles, and therefore bioassays have been developed that assess sub-lethal effects to reproduction in 10 days (Simpson and Spadaro, 2011). Amphipod species are suitable for use in *in situ* tests on sediments in the field (Burton *et al*., 2012).

Mysids are small shrimp-like crustaceans (common name: opossum shrimps) found in both marine and freshwater environments. The majority are omnivores, feeding on algae, detritus and zooplankton and are good candidates for large-scale culture. Mysid species used for estuarine–marine sediment tests include *Americamysis bahia* (North America).

Appendix E provides guidance for undertaking sub-lethal (reproduction) and lethality toxicity tests on whole sediments using the estuarine–marine epibenthic amphipod *Melita plumulosa*.

4.4.4 Meiofauna: Copepods (harpacticoids) and nematodes

Harpacticoid copepods (a particular suborder of copepods, also known as 'harpacticoids') and nematodes (roundworms) are abundant in most sediment environments. Both are considered to be meiobenthic fauna (that is, typically 0.2–2.5 mm in size) and are found in soft-bottom benthic sediments, within the sediment pore waters or adhering to the surface of individual sediment grains.

Harpacticoids are often more abundant than nematodes in silty sands, while nematodes tend to dominate where there is more silt than sand. Harpacticoids may be free-living or create very small burrows; nematodes are free-living, occurring in high densities often exceeding a million individuals per cubic metre. From an ecological perspective, both organisms are important members of benthic communities because they feed on bacteria, algae, diatoms, other meiofauna and plant detritus, and are an important food source for other crustaceans and juvenile fish. Both may ingest fine sediment particles while feeding, potentially resulting in direct exposure to sediment-bound contaminants. During development, they go through multiple larval stages and mid-life stages before reaching full form (for example, nauplii to copepodites to copepod), thus making the species useful for rapid life-cycle whole-sediment bioassays (ISO, 2010; Simpson and Spadaro, 2011; Höss *et al*., 2012). Test endpoints for both organisms typically include survival, reproduction and development/growth.

The nematode *Caenorhabditis elegans* is hermaphroditic and completes its development from laid egg to fertile adult in 3–4 days, making it useful for rapid sub-lethal tests on certain sediments. However, nematode tests can suffer from low tolerance to variations in sediment grain size (Rudel *et al*., 2013). In Europe, *C. elegans* is used for freshwater sediment tests. Commonly used copepod species include *Amphiascus tenuiremis* (North America) and *Nitocra spinipes* (worldwide) for estuarine–marine sediment tests. Appendix F provides guidance for undertaking sub-lethal (reproduction) and lethality toxicity tests on whole sediments using the harpacticoid copepod *Nitocra spinipes*.

4.4.5 Oligochaete and polychaete worms

Oligochaetes (generally freshwater) and polychaetes (marine water) (worms) play significant roles in most sediment environments, particularly through bioturbation of the sediment, breakdown of organic matter, and as prey to other benthic organisms and benthivorous fish. They have a true benthic exposure, with most of them living in constructed tubes or free within the sediment. Most ingest sediment and are subject to potentially high exposure to

sediment-bound contaminants, via ingested sediments, pore water and overlying water, and via dermal contact by direct diffusion of some organic contaminants. In general, oligochaete and polychaete species are more tolerant of contaminants than are other invertebrate classes. Due to their body sizes (or high densities achieved in tests with small species), they are also very useful for bioaccumulation testing. Commonly used polychaete species include *Neanthes arenaceodentata* (North America) and *Arenicola marina* (Europe) for estuarine–marine sediment tests (Bat, 2005; Morales-Caselles *et al*., 2008; Farrar and Bridges, 2011; Ramos-Gómez *et al*., 2011). Commonly used oligochaete species include *Lumbriculus variegatus* (North America) and *Tubifex tubifex* (Europe) for freshwater sediment tests (OECD, 2007; ASTM, 2010).

4.4.6 Bivalves (clams)

Bivalves are an abundant and important component of estuarine and marine benthic communities, and of some freshwater ecosystems (ASTM, 2013b). Bivalves typically bury themselves in the top 2–20 cm of silty or sandy sediments. These 'infaunal' bivalve species feed by using their siphons to draw in organic-rich particles and bacteria and algae from the surface layer, or filtering particles and large volumes of water from the water column. Thus, their food includes organic materials such as bacteria, algae and plant detritus, as well as inorganic sediment materials that are incidentally ingested, and the bivalves are potentially exposed to aqueous contaminants in pore waters, burrow waters and overlying water, and sediment-bound contaminants sourced from the overlying water or from deposited sediments (Griscom and Fisher, 2004; King *et al*., 2010; Campana *et al*., 2013). Bivalves are a principal prey item of many birds, fish and larger invertebrates (and some mammals foraging in sediments) and many species accumulate high concentrations of contaminants in their tissues. They therefore have the potential for trophic transfer of contaminants from sediments and waters to higher trophic levels. The ecological importance of bivalves, their wide distribution and high abundance, their ease of handling and their relative sensitivity to contaminated sediments make them appropriate species for sediment toxicity testing (Ringwood and Keppler, 1998; Keppler and Ringwood, 2002; Duft *et al*., 2003a; King *et al*., 2010; Campana *et al*., 2013). Their significant body sizes make them also very useful for bioaccumulation testing (see Chapter 5) and many species can be used in *in situ* tests on sediments in the field and for general biomonitoring. Appendices G and J describe whole-sediment tests using bivalves.

Bivalve species used for estuarine–marine sediment tests include *Mercenaria mercenaria* and *Mytilus galloprovincialis* (North America) and *Tellina deltoidalis* (Australia). *Lampsilis siliquoidea* is used in for freshwater sediment tests (water-only methods for freshwaters (ASTM, 2013b) have been adapted for conducting whole-sediment toxicity tests with mussels; for example, Besser *et al*., 2013, Wang *et al*., 2013). Oysters have not been used for whole-sediment toxicity tests, but the larval and juvenile stages are used for pore-water testing and are applicable for studying effects of contaminant-release to the water column and from suspended solids (Edge *et al*., 2012, 2014). Appendix G provides guidance for undertaking bioaccumulation and lethality toxicity tests on whole sediments using the bivalve *Tellina deltoidalis*.

Freshwater bivalves possess a remarkable number of physiological, behavioural, life history and anatomical attributes that have made them valuable organisms for use in both contemporary and historical detection of pollution as well as in evaluation of the ecological significance of pollution in freshwater environments (ASTM, 2013b). Australian freshwater bivalves such as *Velesunio angasi*, *Velesunio ambiguus* and *Hyridella depressa* have been used

Table 4.1. Estuarine and marine whole-sediment toxicity tests

Organism	Test species	Duration/ Endpoint	Acute/ Chronic	Reference
Bacteria	*Vibrio fischeri* (Microtox®)	20-min luminescence	Acute	Environment Canada, 2002
Microalga	*Entomoneis* cf *punctulata*	24-h enzyme (esterase) inhibition	Acute	Appendix D; Adams and Stauber, 2004
Amphipod	*Corophium multisetosum*	10-day survival, 21-day fecundity and growth	Acute Chronic	Casado-Martinez *et al.*, 2006; Castro *et al.*, 2006
Amphipod	*Corophium volutator*	28-day survival and growth	Chronic	Scarlett *et al.*, 2007
Amphipod	*Eohaustorius estuarius*, *Leptocheirus plumulosus*, *Rhepoxynius abronius*, *Ampelisca abdita*	10-day survival	Acute	ASTM, 2014; USEPA, 1995; Greenstein *et al.*, 2008
Amphipod	*Gammarus locusta*	28-day survival, fecundity and growth	Chronic	Costa *et al.*, 1998, 2005
Amphipod	*Hyalella azteca* (up to 15 ppt)	10-day, 28-day survival and growth, 42-day survival, growth and reproduction	Chronic	USEPA, 2000; ASTM, 2010
Amphipod	*Leptocheirus plumulosus*	28-day reproduction and growth	Chronic	ASTM, 2014; USEPA, 2001a; Kennedy *et al.*, 2009
Amphipod	*Melita plumulosa*	10-day juvenile survival	Acute	Spadaro *et al.*, 2008; Strom *et al.*, 2011
Amphipod	*Melita plumulosa*	10-day reproduction	Chronic	Appendix E; Mann *et al.*, 2009; Simpson and Spadaro, 2011
Mysid	*Americamysis bahia*	10-day survival	Acute	Kennedy *et al.*, 2009
Copepod	*Amphiascus tenuiremis*	14-day survival and reproduction	Chronic	Chandler and Green, 1996; Kennedy *et al.*, 2009
Copepod	*Nitocra spinipes*	10-day reproduction	Chronic	Appendix F; Perez-Landa and Simpson, 2011; Simpson and Spadaro, 2011
Polychaete worm	*Arenicola marina*	10-day, 21-day survival	Acute	Bat and Raffaelli, 1998; Morales-Caselles *et al.*, 2008
Polychaete worm	*Neanthes arenaceodentata*	20-day survival, 28-day growth	Chronic	Bridges and Farrar, 1997; Farrar and Bridges, 2011

Table 4.1. (Continued)

Organism	Test species	Duration/ Endpoint	Acute/ Chronic	Reference
Bivalve	*Mercenaria mercenaria*	7-day juvenile growth	Sub-lethal	Ringwood and Keppler, 1998; Keppler and Ringwood, 2002
Bivalve	*Tellina deltoidalis*	10-day survival	Acute	Appendix G; King *et al.*, 2010
Bivalve	*Tellina deltoidalis*	30-day survival and growth	Chronic	Campana *et al.*, 2013
Mussel	*Mytilus galloprovincialis*	2-day embryo development at sediment–water interface	Sub-lethal	Anderson *et al.*, 1996; Greenstein *et al.*, 2008
Snail	*Hydrobia ulvae*	48-h post-exposure feeding	Sub-lethal	Krell *et al.*, 2011

Table 4.2. Freshwater whole-sediment toxicity tests

Organism	Test species	Duration/ Endpoint	Acute/Chronic	Reference
Amphipod	*Hyalella azteca*	10- and 28-day survival and growth, 42-day survival, growth and reproduction	Chronic	ASTM, 2010
Amphipod	*Gammarus pulex*	28-day survival, 35-day growth	Chronic	Roman *et al.*, 2007
Nematode	*Caenorhabditis elegans*	96-h survival, growth, reproduction	Chronic	Höss *et al.*, 2012; IOS, 2010
Oligochaete worm	*Lumbriculus variegatus*	28-day survival, growth and asexual reproduction and bioaccumulation	Chronic	OECD, 2007; ASTM, 2010
Oligochaete worm	*Tubifex tubifex*	28-day survival, growth and sexual reproduction	Chronic	ASTM, 2010
Bivalve	*Lampsilis siliquoidea*	28-day survival and growth	Chronic	ASTM, 2013b; Wang *et al.*, 2013; Besser *et al.*, 2013
Chironomid	*Chironomus* sp., *Chironomus riparius, Chironomus dilutus*	10-day larval survival and growth, up to 60-day adult emergence and reproduction	Chronic	USEPA, 2000; OECD, 2004b, 2010; ASTM, 2010

(continued)

Table 4.2. (Continued)

Organism	Test species	Duration/ Endpoint	Acute/Chronic	Reference
Chironomid	*Chironomus tepperi*	5-day larval survival and growth, 10–12-day emergence and sex ratios	Chronic	Appendix H; Kellar *et al.*, 2014
Mayfly	*Hexagenia* sp.	21-day nymphal survival, moulting frequency, growth	Chronic	ASTM, 2010; Harwood *et al.*, 2014
Mayfly	*Ephoron virgo*	21-day nymphal survival and growth	Chronic	Nguyen *et al.*, 2012; Vandegehuchte *et al.*, 2013
Snail	*Bellamya aeruginosa*	10-day survival, 28-day growth	Acute survival Chronic (growth)	Ma *et al.*, 2010
Snail	*Physa acuta*	7-day survival and egg production 21-day larval hatching success and growth (length)	Chronic	Appendix I
Snail	*Potamopyrgus antipodarum*	8-week survival, embryo numbers	Chronic	Duft *et al.*, 2003a,b, 2007
Water flea	*Daphnia magna, Ceriodaphnia dubia*	7-day survival and reproduction	Chronic	ASTM, 2010

to investigate the accumulation of metals in the tissue and shell (Jeffree *et al.*, 1993, Markich *et al.*, 2001). Currently, *V. ambiguus* is being used in caged tests to assess the bioavailability of sediment-bound contaminants in freshwater rivers and lakes dominated by sewage inputs or affected by impacts from acid sulfate soils (Kumar, unpublished).

4.4.7 Chironomids (non-biting midges) and mayflies

Chironomids and mayflies are widely distributed, and have high abundances in many freshwater environments, and their larval or nymph life stages are sensitive to some contaminants. After chironomids hatch from the egg stage, the larvae pass through four larval stages or instars before pupating and eventually emerging as adult midges (typically the life-cycle lasts several weeks or months). The larvae burrow into sediments and construct tubes, open at both ends. They feed on detrital material on the sediment surface. Larvae ventilate their tubes by moving their bodies, thereby facilitating gas exchange during times of low ambient oxygen and also drawing in organic-rich sediment particles for food. Test endpoints include survival, reproduction, growth of midge larvae, and adult emergence and sex-ratios (chronic) (USEPA, 2000; OECD, 2004a,b, 2010; ASTM, 2010; Nguyen *et al.*, 2012; Kellar *et al.*, 2014).

The nymphal stages of mayflies usually last for one year. As detritivores, most of the tube-dwelling species are exposed to contaminants via ingestion of sediment and general detritus, as well as via pore water and overlying water. Most mayfly toxicity tests use field-collected organisms or eggs, because of the long time to maturity. Test endpoints usually include survival, moulting, growth and avoidance behaviour (ASTM, 2010).

Chironomid species used in freshwater sediment tests include *Chironomus riparius* and *C. dilutus* (North America and Europe; USEPA, 2000; ASTM, 2010) and *C. tepperi* (Australia; see Appendix H). Mayfly species used in freshwater sediment tests include *Hexagenia* sp. (North America) and *Ephoron virgo* (Europe) (ASTM, 2010; Harwood *et al*., 2014). Some species are suitable for use in *in situ* tests on sediments in the field (Chappie and Burton, 1997; Sibley *et al*., 1999; Castro *et al*., 2003). No species of midge or mayfly is routinely used for estuarine–marine sediment tests because these primarily freshwater taxa have limited salinity tolerance.

4.4.8 Gastropods (snails)

Several gastropod (snail) species are widely distributed and have relatively high abundances in many freshwater and marine environments. Due to their abundance and size, snails are important in many food webs, both as decomposers and as consumers (most are herbivores and very few are carnivores). Many species tolerate wide variations in salinity and temperature, and some are suitably sensitive to contaminants for use in ecotoxicology testing (Duft *et al*., 2003a,b, Zalizniak *et al*., 2009; Ma *et al*., 2010). Snails are also ideal for bioaccumulation programs or as a source of tissue samples for molecular ecotoxicology techniques. Many species are deposit feeders, ingesting sediment particles and organic detritus, algae and bacteria in the sediment or other organic carbon-rich substrates, and are expected to be highly exposed to sediment-associated contaminants as a consequence. Commonly used snail species include *Hydrobia ulvae* and *Valvata piscinalis* (Europe) for estuarine–marine sediment tests, and *Potamopyrgus antipodarum* and *Physa acuta* and *Lymnaea stagnalis* for freshwater sediment tests. Appendix I provides guidance for undertaking sub-lethal (reproduction) and lethality toxicity tests on whole sediments using the snail species *Physa acuta*.

4.5 Toxicity endpoints

Test endpoints are usually referred to as acute or chronic, although sub-lethal or lethal are also used. Acute toxicity typically refers to an adverse effect that occurs as the result of a short period of exposure to a chemical, relative to the organism's life span. Chronic toxicity typically refers to an adverse effect that occurs as the result of exposure to a chemical for a substantial proportion of the organism's life span or an adverse sub-lethal effect on a sensitive early life stage. A substantial proportion of an organism's lifetime would typically be greater than 10% (Newman, 2010). As a general rule, acute toxicity tests are conducted for at least 48 h (algal growth rate, avoidance, post-exposure feeding endpoints), and for 4 days to 10 days for other species (survival). Chronic sediment toxicity tests should have a duration of >10 days, while durations of 28–42 days or up to 60 days have been used for assessing longer-term effects on survival, growth and reproduction of amphipod, bivalve and worm species, or emergence of chironomids (USEPA, 2000; OECD, 2004b; ASTM, 2010, 2014).

Organism survival has been the most commonly used endpoint for tests applied to sediment quality evaluations, and usually this is an acute endpoint. Juvenile life stages are more sensitive than adult life stages. Sub-lethal organism-level endpoints such as growth (for example, change in mean weight), biomass (for example, average weight of individual

(mg/individual) or total mass of organisms in a replicate (mg biomass/replicate)), development (for example emergence, for insects), and reproduction (for example, number of offspring produced) are generally more sensitive and more relevant for assessing risk. These sub-lethal responses are usually considered as chronic endpoints and provide greater information on the potential for long-term effects at the individual and population level. Robust sub-lethal tests are increasingly available and some good comparisons have been made of many of these (Greenstein *et al.*, 2008; Kennedy *et al.*, 2009; Simpson and Spadaro, 2011).

The biological and ecological relevance of the test endpoints should be well understood in order to apply them effectively for making decisions on management options for contaminated sediments. Physiological and biochemical responses (for example, changes in biomarkers or gene expression) and energy balances are being increasingly proposed as suitable test endpoints; however, only a few biomarker responses have been demonstrated as providing greater sensitivity or less variability than the well-established sub-lethal endpoints in whole-sediment tests (Simpson and Spadaro, 2011; Edge *et al.*, 2014). Direct links between lysosomal instability and impaired reproduction have been demonstrated as being more sensitive for some species (Edge *et al.*, 2012). Biomarkers have also been applied to assess the impact of dredged materials. Most of the studies focus on determining the activity of biotransformation enzymes, antioxidant enzymes and biochemical indices of oxidative damage (Ingersoll *et al.*, 2005; Martins *et al.*, 2012; Tsangaris *et al.*, 2014). Like these traditional biomarkers, molecular approaches, such as ecogenomics or ecotoxicogenomics, may prove to be a suitable tool for facilitating the interpretation of bulk sediment toxicity data, because the molecular response of an organism is arguably more sensitive and more specific than the response at higher levels of organisation.

Pragmatically, the purpose of eco(toxico)genomics is to identify gene and/or protein classes that are switched on or off upon exposure, thus making it possible to detect molecular fingerprints specific to the bioavailable fraction of the chemical (Custodia *et al.*, 2001; Menzel *et al.*, 2005; Biales *et al.*, 2013; Hook *et al.*, 2014). However, as discussed in Chapter 6, the ecological relevance of such approaches can really only be seen by linking these responses (for example, through adverse outcomes pathways) to organism-level (for example, reproductive output) and/or population-level responses (for example, population growth).

Endpoints expressed at lower levels of biological organisation (for example, molecular responses) are frequently more variable (highly sensitive to many non-contaminant factors), and this often leads to less well defined concentration–response relationships and weaker links to potential impacts on populations. While that can inhibit the use of some molecular-based endpoints, potential advantages include the potential for greater specificity in determining causes of toxicity (toxicity identification evaluation (TIE); USEPA, 2007) through specific responses to individual contaminants (Biales *et al.*, 2013).

Behavioural responses, such as changes in post-exposure feeding (Moreira *et al.*, 2005; 2006; Soares *et al.*, 2005; Krell *et al.*, 2011; Rosen and Miller, 2011) and avoidance (Araújo *et al.*, 2012; Ward *et al.*, 2013a,b) have also been used for toxicity assessments, but few standardised methods are currently available.

4.6 Toxicity-modifying factors

Total contaminant concentrations are often poor predictors of the toxicity of contaminants in sediments (Simpson and Batley, 2007). Similar concentrations of a contaminant in units of mass per mass of sediment (dry weight) often exhibit a range of toxicities in different sediments. Evaluating effects concentrations for specific contaminants in sediments requires knowledge of factors influencing their bioavailability (see Chapter 3 Section 3.5).

Partitioning of a contaminant between water and sediment may depend on many factors including aqueous solubility, pH, redox potential, affinity for sediment organic carbon and dissolved organic carbon, grain size of the sediment, sediment mineral constituents (oxides of Fe, Mn and Al), and the quantity of acid volatile sulfide (AVS) in the sediment. Although certain contaminants are highly sorbed to sediment, these compounds may still be available to the biota, thus creating a need to assess exposure and effects from both the dissolved and the particulate phases.

Measurement and reporting of sediment properties and contaminant partitioning significantly improve toxicity assessments. The accuracy of the toxicity assessment will also be influenced by the procedures used for the collection, transport, handling and storage of sediment collected from field sites, and the manipulation of sediments in the laboratory (see Chapter 2 Sections 2.3, 2.5, 2.6 and USEPA, 2001b; ASTM, 2008b).

There are several other factors that affect a test organism's response, apart from those related to sediment-associated contaminants. They include effects of sediment heterogeneity, sediment manipulations, temperature, light, food availability (sediment nutrition) and organism mobility. These factors may result in significant toxicity (negative effects relative to control responses) when contamination is low, greater toxicity than expected, or no (or less) observed toxicity when contaminants are present at concentrations expected to cause great toxicity (Bridges *et al*., 1997; Spadaro *et al*., 2008).

The heterogeneity of natural sediments often makes it difficult to extrapolate from laboratory studies to the field. As discussed in detail earlier (Chapter 2 Section 2.6), manipulations of sediments (collection, handling and storage) may alter organisms' exposure to contaminants by changing the physical, chemical or biological characteristics of the sediment, resulting in losses, transformations or changes in the partitioning of the contaminant.

Differences in the temperature used for testing, especially in the field (particularly for tropical or polar samples), will affect contaminant solubility, partitioning and other physical and chemical characteristics.

Photo-induced toxicity has been observed to be an important factor in controlling the toxicity of some contaminants associated with sediment (for example, certain PAHs (Hatch and Burton, 1999; Fathallah *et al*., 2012)). The light conditions used during laboratory tests may need to reflect conditions at the field site (for example, at a certain water depth or sun-exposure on a mud flat).

Motile organisms may be able to avoid exposure to contaminants in the field, and that may need consideration in the final assessment process (Ward *et al*., 2013b). It is often observed from toxicity assessments that organisms can survive but not reproduce in contaminated sediments (Simpson and Spadaro, 2011). Also, manipulative field experiments that deploy contaminated sediments in clean environments (see Chapter 7 Section 7.4) have shown that organisms can recolonise and reside in these sediments when the organisms are recruited from cleaner surrounding environments, but they may not necessarily be able to reproduce in the same sediments (Hill *et al*., 2013).

4.6.1 Non-contaminant stressors influencing test results (ammonia, sulfide, particle size)

A range of non-contaminant stressors can affect the toxicity test responses during exposure to sediment and it is important to consider these when interpreting the results. Common non-contaminant stressors include salinity (to which organisms have varying tolerance), ammonia or sulfide in overlying water and pore water, and sediment properties (particle size). While these stressors are naturally occurring, the effects of both ammonia and sulfide

concentrations can be greatly exacerbated by anthropogenic inputs such as nutrients, and they may become major toxicants as a result.

During toxicity tests, the physico-chemical characteristics of the test sediment and water should be within the known tolerance limits of the selected test organism (Moore *et al*., 1997). Control and reference sediments should have matching ranges of particle size and salinity. Ammonia and sulfide concentrations in pore waters and overlying waters should be measured and the exposure in the overlying waters of laboratory-based toxicity tests considered during the test design. In some cases, modification of test design may be appropriate for achieving the desired properties; for example the rate of exchange of overlying water will influence ammonia concentrations (Word *et al*., 2005). However, most tube-building benthic invertebrates typically circulate oxygenated water through their burrows thus reducing exposure to pore-water ammonia and hydrogen sulfide. Toxicity identification evaluation (TIE) procedures can be used to assist in identifying where ammonia is a major toxicant (see Section 4.10 below).

In very sandy sediments, addition of food may be necessary for organisms to maintain their growth and development within predictable ranges during long-term exposures. However, addition of uncontaminated food may lead to selective feeding and therefore reduce the exposure of the test organisms to sediment-bound contaminants; this needs to be evaluated (Åkerblom and Goedkoop, 2003; Spadaro *et al*., 2008).

In sediments, the presence of indigenous organisms can prove problematic, especially if they are predatory. To prevent interference from larger predators, sediments may be sieved to remove these before testing. For small predators, one solution has been to hold sediments longer before testing, letting them become anaerobic to kill off indigenous organisms. If such manipulative procedures are necessary, it is important to undertake analyses afterwards, to determine whether the sediments suitably represent the sediments as collected from the field (after a re-equilibration period); for example, to test that pore-water pH and AVS concentrations are similar to those existing before manipulation.

4.7 Test design and quality assurance

4.7.1 Test conditions

Organisms display a wide range of tolerances to natural physico-chemical variables (for example, temperature, salinity, pH, sediment particle size) and variations in these parameters and the test conditions (for example, water renewal rates, light/dark conditions) during tests may influence the test endpoints. While often it is ideal to have test organisms that can tolerate wide variations in physico-chemical variables so that the test can be applied widely, most organisms have narrow tolerance limits for some variables. Biological factors also can modify endpoints; for example, organism density, food quantity and quality requirements, and potential cannibalism (particularly of juveniles). As a consequence, it is necessary to evaluate, specify and stay within tolerance ranges of test organisms. It is also useful to know the optimal ranges for any test. General specifications for any test will include the target ranges required. For example, for a whole-sediment test on a marine organism, these specifications and ranges might be:

- test duration (for example, 10 days);
- temperature (for example, 21 ± 1°C);
- light intensity and photoperiod (for example, often tests are conducted within a controlled room or environmental chamber, 12-h dark : 12-h light (for example,

1–10 μmol photons/s/m^2 for an algal test and 50–70 μmol photons/s/m^2 for an algal culture (Appendix D));
- salinity (for example, 30 ± 2‰);
- aeration requirements (for example, 85% saturation of dissolved oxygen in overlying water throughout the test);
- test chamber size and material (for example, 250 mL glass beaker);
- total test volume (for example, 250 mL);
- sediment weight or volume (for example, 100 g, 50 mL);
- overlying water (for example, uncontaminated seawater collected from a clean site, filtered to 0.45 μm and adjusted to test salinity);
- overlying water volume (for example, 180 mL);
- sex/age/state/size of test organisms (for example, gravid females 5–10 mm length);
- number of test organisms per test chamber (for example, 15);
- number of replicate chambers per sample (for example, 4 minimum, possibly extras for chemistry monitoring);
- feeding regime (for example, no feeding during 96-h test; feeding twice weekly 10 g fish food per animal during 40-day test);
- overlying water renewal (for example, daily renewal, or continuous renewal);
- control sediments and reference sediments (for example, uncontaminated sediment with similar physico-chemical parameters, such as grain size, pore-water salinity and pH, to the test sediment); and
- endpoint (such as reproduction, or survival and tissue residues).

Further examples of test conditions are provided in the methods described in the appendices. Parameters measured during tests (for example, temperature, salinity, dissolved oxygen) should be reported along with the results. Control charts that record these parameters and endpoint results such as survival, reproduction and growth of test organisms in control and reference toxicant tests are useful for monitoring both short-term (for example, with a testing program) and long-term (between testing programs) test performance. Some specific test considerations are discussed further below.

4.7.2 Test organism handling and acclimatisation

Whether sourced from cultures or field-collections, organisms of a similar life stage, age and size should be used in sediment toxicity tests. If field-collected, benthic invertebrates may often be less stressed if allowed to rebury in sediment (for example, 2–4 cm depth of sieved sediment) before being transported in containers with overlying water from the collection site. Considering and ensuring appropriate densities of organisms should avoid additional stress. Field-collected organisms should be given sufficient time (for example, 2–10 days) to acclimatise to laboratory testing conditions for example, before test commencement (ASTM, 2012), with regular replacement of overlying water to maintain sufficient dissolved oxygen. If salinity adjustment is required (for example, when using field organisms collected from a salinity different to that used in the tests (Hyne and Everett, 1998)), this should be done slowly, allowing at least a day for acclimatisation at each salinity (2‰ incremental increase) until the desired test salinity is reached. Any dead or visibly unhealthy organisms, or organisms that behave atypically, should be removed and discarded before the toxicity test.

4.7.3 Sediments

The types of sediments collected for toxicity testing depend on the study aims. For most monitoring and toxicity assessment studies where historical contamination is not an issue,

the upper few centimetres of sediment are the horizon of interest (for example, 0–2 cm, or 0–10 cm surface sediments). Most infaunal organisms are found in the upper 10 cm (except for deep burrowers) and the use of various grab sampling devices is discussed in Chapter 2 Section 2.3 and in various review documents (for example, Environment Canada, 1994; USEPA, 2001b). Alternatively, sediment cores can be collected and either homogenised or sectioned to provide sediment from a particular depth for toxicity testing. Whatever sampling method is used, anoxic sediments will become oxidised during collection, transport and preparation (for example, by sieving) for toxicity testing (Simpson and Batley, 2003). Oxic sediments are appropriate for toxicity testing because most sediment infauna reside in the top oxic layer or irrigate their burrows with oxygenated overlying water.

4.7.4 Quality assurance and control (QA/QC)

Control sediments

Control sediments are used to monitor the performance of tests over the short term (for example, during a testing program) and long term (between testing programs). Sediment tests should include a control sediment (sometimes called a negative control) that is essentially free of contaminants. This is used to assess the acceptability of the test, to provide evidence of test organism health, and, along with reference sediments, can be used as a reference for interpreting effects in the test sediments. The endpoint responses in control sediments are typically those used to prepare control charts to track the performance of bioassays over time.

Two types of control sediments can be used:

(i) natural field-collected sediments; and
(ii) prepared sediments such as formulated sediments (also called reconstituted, artificial or synthetic sediments).

Natural (field) control sediments should be collected from any uncontaminated site. Formulated control sediments are not frequently used; details of several formulations and procedures for their preparation are given elsewhere (Environment Canada, 1995; USEPA, 2000, 2001b; OECD, 2007, 2010; ASTM, 2010). The physico-chemical properties of control sediments should be determined and reported; for instance, grain size, total organic carbon, AVS and background concentrations of trace elements. Pore-water pH, salinity, metal and ammonia concentrations in the control sediments should also be determined. Field-collected control sediments should be stored at 4°C in sealed containers in the dark. During storage, the pH and redox potential and the concentration of volatile or redox-sensitive constituents, such as pore-water ammonia, sulfide, iron and manganese, and AVS, are likely to change because of changes in oxygen ingress and bacterial activity. Changes in these parameters may influence the bioavailability of contaminants and toxicity to test organisms and it may be necessary to measure them in control sediments that have been stored for long periods (for example, >3 months) before use.

Reference sediments

Variability in test endpoints may be attributable to variations in non-contaminant factors that occur within the assessment area. This situation can be explored by testing reference sediments, which are usually collected from sites near the contaminated assessment site but at locations known not to contain the contaminants of interest. Use of reference sediments provides a site-specific baseline for evaluating the effects data. Reference sediments are

useful for interpreting toxicity tests; parallel tests using reference sediments can help estimate the relative contributions of natural stressors and other anthropogenic toxicants beyond the chemical(s) of interest in the test sediment (USEPA, 2000; ASTM, 2010; Wang *et al.*, 2013). In situations where there is expected to be a gradient of contaminants away from a point source (for example, away from an industry), then sediments beyond this gradient may act as useful reference sediments.

Reference toxicants

A reference toxicant (sometimes called a positive control) is a chemical that is used in toxicity testing to make comparisons between inter- or intra-laboratory test results. Reference toxicants are intended to provide a general measure of the reproducibility (precision) of a toxicity test method over time. If individual test results fall outside established limits (for example, LC50 ± 2 standard deviations), an investigation into the source of the variability is triggered. Inclusion of a reference toxicant in each toxicity test may help to determine variability in organism health in different batches of test organisms, and differences in laboratory water quality and the operational consistency of testing personnel. Control charts should be used to track the performance of bioassays over time.

For sediment tests, reference toxicants may be used in either water-only exposures or in spiked sediment tests. Because of the ease of conducting water-only exposures and the relative speed with which results can be obtained, reference toxicant tests using water-only exposures have traditionally been used in conjunction with whole-sediment tests. For a 4- to 7-day static test, a range of concentrations of a toxic metal (for example, copper) or organic toxicant (for example, sodium dodecyl sulfate) may be suitable. Ideally, these should be initiated using the same batch of field-collected test organisms and within a few days of commencing the whole-sediment tests. For organisms from cultures, reference toxicant tests may be undertaken on a routine basis, rather than for each batch of test organisms used in tests. If a sediment is spiked with a reference toxicant, then careful consideration should be given to the spiking and equilibration period (Simpson *et al.*, 2004) (see Chapter 2 Section 2.9 and Appendix B).

Test acceptability criteria

The criteria for determining test acceptability (specific to each toxicity test species) are an essential component of good quality assurance procedures. For most whole-sediment tests, survival in the controls would have to be at least 70–90% (ASTM 2010, 2014), and similar acceptability criteria may also exist for reproduction (Simpson and Spadaro, 2011). In addition, water quality parameters such as pH, salinity, dissolved oxygen and temperature should be within set limits. Reference toxicant tests using either water-only exposures or spiked sediments should give EC50/LC50 values within ± 2 standard deviations (s.d.) of the mean value obtained from quality control charts specific to that test organism and test conditions.

If sediment disturbances during sampling and manipulation before testing are suspected of altering the concentrations or bioavailability of contaminants (for example, if sediments containing volatile organics are sieved before tests), some discussion should be included on how this may influence the outcome and interpretation of the toxicity test results. For example, if AVS decreased, then metals may have been more bioavailable in the manipulated sediments.

4.8 Toxicity test data analysis and interpretation

The objective of data analysis is to quantify contaminant effects on replicate groups of exposed test organisms and to determine if these effects are significantly different from those

occurring in the control sediments (ASTM, 2013a). Similar comparisons are made with the results from reference sediments. Statistical endpoints such as percentage survival (mean ± s.d.) or the percentage of impairment (for example, growth, reproduction) should be calculated for each treatment and compared to the reference sediment or control sediment data.

After testing the data for normality and homogeneity of variance, pairwise comparisons of treatment versus control (or reference) data are made (for example, by Student's *t*-test, or ANOVA followed by Dunnett's or Tukey's tests). If the requirements for normality and homogeneity of variance are not met, the data can be transformed and re-tested. If the data still fail, a non-parametric test such as the Wilcoxon Rank Sum test can be used for the statistical comparison. Details of a range of statistical methods for data analysis are provided elsewhere (OECD, 2006; ASTM, 2014, 2010, 2013a). The American Society for Testing and Materials (ASTM) has published a guide for the selection of resident species as test organisms (ASTM, 2012), and also a detailed list of statistical methods for toxicity tests, including possible flow charts, to assist with choosing suitable analysis methods (ASTM, 2013a).

As part of the quality assurance procedures (Section 4.7.4 above), test acceptability criteria must be applied to the results achieved for the controls and reference toxicants (for example, within acceptable ranges) and to sediment and water physico-chemical parameters (for example, water pH, salinity, ammonia). If all aspects of the test meet the appropriate acceptability criteria, data interpretation can then be carried out. In order to interpret any toxicity detected, it is important to ensure that the tolerance ranges of the test organism are not exceeded in relation to physico-chemical characteristics such as dissolved oxygen, pH, salinity and particle sizes in the test sediments, pore waters and overlying waters. Any influence of naturally occurring or anthropogenically exacerbated ammonia and sulfide concentrations must also be considered.

4.8.1 Determination of toxicity

A range of criteria can be used to determine whether or not the test sediment is toxic. For amphipod acute tests, Environment Canada recommends the use of pass/fail criteria based on mortality being significantly different and exceeding that of the reference sediment by more than 20%. In the absence of suitable reference sediment, the test sediment is judged to be toxic if mortality is more than 30% higher than mortality in the control and differs significantly from it (Environment Canada, 1998). For sub-lethal sediment toxicity tests with the amphipod *Melita plumulosa* and copepod *Nitocra spinipes*, a sediment has been classified as toxic when the mean reproductive output of the organism in the test sediment was less than in the control by 15% for *M. plumulosa* or 25% for *N. spinipes*, and the difference was statistically significant (*t*-test, $P < 0.05$) (Simpson and Spadaro, 2011). For the benthic algal test, a sediment has been considered toxic when there was more than 20% inhibition in enzyme activity compared to the reference (or control) sediment (that is, activity was <80% of the activity in the control), and the difference was statistically significant ($P \leq 0.05$) (Adams and Stauber, 2004).

4.8.2 Calculation of effects thresholds (LC50, EC10)

Where there is a gradient of exposure concentrations, without co-variation of other contaminants, it may be possible to calculate specific effect thresholds. These may be necessary for the construction of species sensitivity distributions (SSDs) for sediment contaminants (Simpson *et al.*, 2011; Vangheluwe *et al.*, 2013) or to establish site-specific management limits (Simpson *et al.*, 2013; Besser *et al.*, 2014). The concentration–response parameters

desired are generally the lower thresholds of effects. These are generally the concentrations that cause a 10% effect (EC10) or 10% lethality (LC10), or they are the no-observed-effect concentrations (NOECs), or the no-effect concentrations (NECs; Van Der Hoeven *et al.*, 1997; Fox, 2010). There is a general move away from the use of NOECs, which are based on hypothesis testing, towards the use of ECx or LCx values (typically with x = 10) (Batley *et al.*, 2014). If NOECs are used, then these should be bounded (that is, have tested concentrations below, and a lowest-observed-effect concentration (LOEC) not far above). The ECx variables are calculated using standard regression-model extrapolation/interpolation methods (for example, probit, logit, Weibull, or some generalised linear models), or using NECs by Bayesian methods (Fox, 2010) and NOEC/LOEC values by hypothesis tests for significant difference from the control (OECD, 2006). For specific guidance on the use of SSDs to derive new guidelines or site-specific management limits, the extensive water quality guidelines literature should be consulted (Batley *et al.*, 2014; Warne *et al.*, 2014).

4.9 Bioanalytical approaches

Sediments can contain mixtures of biologically active compounds with mechanisms of action that differ from those of many commonly assessed classes of contaminants such as metals and hydrophobic organic contaminants. Endocrine-disrupting chemicals (EDCs) are a class of chemical contaminants with the potential to interact with the endocrine system (UNEP/WHO, 2013). Compounds well recognised as EDCs include polychlorinated biphenyls (PCBs), alkylphenols, polybrominated diphenyl ethers (PBDEs) and a variety of phthalates. A great many other insecticides, fungicides, industrial chemicals (including tributyltin (TBT)), and many metals and metalloids also contribute to EDC-related effects (UNEP/WHO, 2013; Vandenberg *et al.*, 2012). An EDC has been defined as 'an exogenous agent that interferes with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body that are responsible for the maintenance of homeostasis, reproduction, development and/or behavior' (USEPA, 1997). In the aquatic environment, the EDCs present are very diverse in terms of chemical nature and origins (Vos *et al.*, 2000) and their analytical determination is often difficult. Moreover, for several compounds, the endocrine-disrupting potency is unknown. Thus, an analytical determination of total endocrine-disrupting activity of the complex mixture is not possible, and development of sediment quality guideline values based on chemical measures is not feasible. Some integrative measures of exposure are needed to determine the endocrine-disrupting potential of complex mixtures. Several basic mechanisms exist for endocrine disruption, including receptor-mediated mechanisms such as those of ligand agonists (which are drugs that bind to and activate a receptor) and antagonists; inhibition of synthesis; and inhibition or acceleration of metabolism of endogenous hormones. Despite their various structures, several chemicals can elicit effects via a mode of action similar to estrogen (Vandenberg *et al.*, 2012; UNEP/WHO, 2013).

Because of the large diversity of EDCs and their effects, bioanalytical approaches using mechanism-based biological screening tools have emerged to monitor such substances in complex environmental mixtures (Eggen and Segner, 2003). Rather than using whole sediments, these approaches typically use water or organic extracts, making them prone to the issues associated with pore-water tests. *In vitro* recombinant cell bioassays, in which a reporter gene is under the control of receptor binding, enable estimation of the total receptor-mediated activity of samples and also account for possible interactions between compounds in the mixture. *In vitro* assays provide robust, sensitive and specific bioassays to

screen and quantify endocrine activities in environmental samples. A look at the structural diversity of several hundreds of chemicals suspected to act as EDCs illustrates the necessity to cover the effects in an integrative manner with bioassays, because EDCs are more easily identified by their estrogenic effect than by their chemical structure. It is only possible to make a prediction of the structural characteristics in exceptional cases, and chemical analyses cannot predict antagonistic and synergistic effects of different EDCs. Moreover, a screening for all known EDCs in environmental samples would be very costly and impractical for environmental monitoring purposes. Combining bioanalytical tools with powerful chemical analyses within integrated approaches permits scientists, regulators and managers to identify key toxicants to be monitored in the environment. By using such bioanalytical approaches, the occurrence of EDCs and their effects on aquatic organisms have been reported in several industrialised countries (Vethaak *et al.*, 2005; Jobling *et al.*, 2006).

Much attention has been directed to identifying estrogenic chemicals, that is, those capable of binding to and activating the estrogen receptor (ER), because many of the effects reported in wildlife appear to be a consequence of feminisation of males (Sumpter, 2005). However, many environmental contaminants can interfere with other molecular targets involved in the regulation of the endocrine system. These include other nuclear receptors such as androgen receptors (AR), pregnane X receptors (PXR) (Mnif *et al.*, 2007) and steroidogenesis enzymes such as aromatase (Laville *et al.*, 2006). EDCs can also act via indirect mechanisms such as (anti)estrogenic effects mediated by aryl hydrocarbon receptor (AhR) ligands through an ER/AhR cross-talk dependent mechanism (Ohtake *et al.*, 2003, 2007). Therefore, a comprehensive assessment of EDC hazard should take into account the ability of chemicals to interfere with different targets of the endocrine system (Houtman *et al.*, 2004).

Recombinant yeast-based and chemically activated luciferase gene expression (CALUX®) reporter gene assays are being used to measure the endocrine potential of emerging contaminants including pharmaceuticals and personal-care products in environmental river water and sediment samples (Murk *et al.*, 1996; Kumar *et al.*, 2012; Li *et al.*, 2013, Bain *et al.*, 2014). A battery of assays following the CALUX® approach have been used: ER CALUX®/anti-ERCALUX® for estrogens and pseudo-estrogens, AR CALUX®/anti-AR CALUX® for androgen hormones, PR CALUX®/anti-PR CALUX® for progesterone-like activity; GR CALUX®/anti-GR CALUX® for glucocorticoid-like activity, and PPARgamma2 CALUX® for peroxisome proliferator-activated receptor-like activity (Bain *et al.*, 2014).

There are a range of additional cell-based bioassays that assess receptor–mediator endpoints. For example, DR-CALUX® and AhR-CAFLUX (chemically activated fluorescence expression) target dioxin and dioxin-like compounds based on Ah-receptor induction (Houtman *et al.*, 2006; Tang *et al.*, 2013) and AREc32 (a reporter gene assay) targets electrophilic compounds acting on the Nrf2-mediated oxidative stress pathway (Wu *et al.*, 2012).

These *in vitro* methods are suitable for screening purposes because of several interesting properties, one of which is the potential to apply high-throughput methods and reduce costs relative to animal experiments. However, it is necessary to point out the limitations of the bioanalytical tools, and to establish the biological/ecological relevance of the endpoints. The *in vitro* assays do not account for pharmacokinetics, tissue distribution and biotransformation processes that may be observed in *in vivo* animal models. In addition, contaminants that exhibit toxicity through metabolic activation could be overlooked in the *in vitro* bioassays.

4.10 Toxicity identification evaluation (TIE) for whole sediments

Use of toxicity identification evaluation (TIE) procedures for dissolved toxicants in natural waters is now well established (for example, USEPA, 1996). Such procedures are not directly

transferrable to toxicants in sediments because of the added complexity associated with the solid phase. TIE procedures for sediments are more limited in their application (USEPA, 2007), but identification of toxicant classes affecting aquatic ecosystems, in particular the health of benthic organisms, is becoming an increasingly important part of sediment quality assessment programs (NFESC, 2003; USEPA, 2007; Ho *et al*., 2009; Ho and Burgess, 2009, 2013; Burgess *et al*., 2011).

The procedures involve manipulation of sediments or sediment components (for example, pore waters) to remove or alter bioavailability of an individual toxicant (for example, ammonia) or class of contaminants (for example, hydrophobic organics, metals) from the pore water. This then allows identification of an individual toxicant or class of toxicants responsible for the observed toxicity (Ankley and Schubauer-Berigan, 1995; Ho *et al*., 2002, 2004; Burgess *et al*., 2003, 2004, 2007, 2011; USEPA, 2007).

In the USEPA (2007) framework, TIE methods are divided into three phases: characterisation, identification and confirmation. The procedural differences between toxicity test methods for pore waters and whole sediments (including sample preparation) make it desirable to have TIE procedures that can be applied to whole sediments. Whole-sediment TIEs are expected to be more 'accurate' and provide more realistic exposure pathways for organisms. To date, three major separations have been applied:

- Addition of a metal-chelating resin to sediments has been found to be a useful whole-sediment TIE method that reduces the concentration and toxicity of metals, but has only minor effects on the toxicity of ammonia and non-polar toxicants present in the sediments (Burgess *et al*., 2000). Furthermore, the resin and accumulated metals are able to be isolated from the test system following exposures, allowing for the initiation of the identification stage of the TIE procedure.
- Removal of ammonia toxicity has been achieved in whole-sediment toxicity tests by addition of the marine algae *Ulva lactuca*, or zeolite (Besser *et al*., 1998; Ho *et al*., 1999; Burgess *et al*., 2004).
- Addition of powdered coconut charcoal has been found effective to remove the toxicity of organic contaminants such as PAHs, PCBs and pesticides (Ho *et al*., 2004).

It is expected that future TIE methodologies will be improved by the incorporation of knowledge of contaminant exposure pathways (for pore waters or whole sediments) (Simpson and King, 2005, Rainbow, 2007), and of species-specific sensitivity to selected contaminants (Borgmann, 2000; King *et al*., 2006) and the use of multiple species in the experimental design (Isidori *et al*., 2003). Recently, genomic biomarkers have been applied to TIEs with the aim of improving the identification of specific toxicants (Biales *et al*., 2013; Hook *et al*., 2014).

TIE procedures for pore waters are generally cheaper, faster, and easier to conduct than the current 'developmental' whole-sediment TIEs (Carr and Nipper, 2003). Current advantages of using pore-water TIEs include:

- the ability to use test organisms that are not compatible with a sediment matrix;
- that pore waters are considered a major route of exposure for many toxicants;
- there is a good understanding of how sample manipulation affects sample chemistry;
- the availability of established water TIE testing methods.

However, several factors disadvantage pore-water TIE procedures, making it desirable to have whole-sediment TIE methods. For example, many of the sample manipulation artefacts that affect contaminant speciation and bioavailability (discussed in Chapter 2 Section 2.6) also affect the results of TIE procedures.

4.11 *In situ* testing

While laboratory studies provide an ability to control environmental factors, which facilitates the identification of cause and effect relationships, they lack the complexity and variability of the natural environment. Multiple sources of stress may have impacts on individuals, populations and communities in the field but are impractical or impossible to replicate in laboratory-based assays. This may result in laboratory-based effects assessments providing a poor measure of field-based exposures and effects (Chappie and Burton, 2000; Burton *et al.*, 2005, 2012). This situation is most likely to occur when exposure dynamics and interactions occurring at the field site cannot be correctly mimicked in the laboratory (examples include natural rates of photo-induced toxicity of PAHs, stormwater runoff events resulting in fluctuating exposures, and salinity changes).

In situ tests may be defined as environmental measurements that are taken in the field, without removal of a sample to the laboratory, thus minimising manipulation. *In situ* measures of effects may more accurately represent the effects on single organisms or populations in the field. Advantages of *in situ* testing over laboratory-based tests include:

- reduction of artefacts related to sediment sampling and processing;
- better representation of both biotic and abiotic factors and stressors that affect contaminant exposure dynamics and uptake pathways (especially natural food availability);
- access to near-real time effects data;
- ability to assess temporal or episodic toxicity.

Disadvantages of *in situ* testing may include:

- the logistics associated with placement and retrieval of *in situ* chambers (in deep sites) and integrity during placement (for example, vandalism, high-energy sites);
- organism stress if transported to the field site;
- the rate of acclimatisation of organisms to field conditions;
- artefacts associated with chamber burial and organism suffocation, mesh fouling due to suspended solids, low dissolved oxygen levels, food availability and predation;
- changes to contaminant dynamics at the sediment–water interface due to chamber deployment;
- limited availability of reference sites that suitably represent the conditions at the test site;
- the complexity of data interpretation.

In situ toxicity testing usually involves the use of mesocosms containing single species to assess toxicity of undisturbed field sediments, but may be applied to transplanted or spiked sediments. The *in situ* test design will depend on the study objectives, but may use a variety of organisms, chamber devices and study designs in marine and freshwater environments (Burton *et al.*, 2005, 2012; Rosen *et al.*, 2012; Ferrari *et al.*, 2014). The test design will strongly influence the test organism's exposure to stressors, especially water flow, light, suspended solids concentrations, food availability and interactions with predators. The use of appropriate controls and reference conditions is also an important consideration, and artificial sediments (formulated controls or spiked sediments) may be useful for investigating the effects of specific contaminants or factors such as food availability. The monitoring of water quality conditions (dissolved oxygen, salinity, temperature, suspended particulate matter) within and around *in situ* enclosures (continuously where possible) is often essential for interpretation of *in situ* test results.

Owing to the site-specific nature of *in situ* testing, standardised methods are not readily available, although several attempts have been made for specific environments; for example, using amphipods (*Leptocheirus plumulosus*), polychaetes (*Neanthes arenaceodentata*) and chironomids (Burton *et al*., 2012; Rosen *et al*., 2012; Ferrari *et al*., 2014). Incorporation of results from *in situ* tests in the weight-of-evidence decision-making process is desirable.

4.12 Examples of sediment toxicity assessments

To illustrate the common application of sediment ecotoxicology assessments, two scenarios are considered: (i) a contaminated estuarine environment in which sediments are to be disturbed for urban or industrial development potentially by dredging and disposal in a new location; and (ii) a body of freshwater that has potentially been affected by a range of organic and metal contaminants.

4.12.1 Harbour sediment assessment case study

The issue

Evaluation of the impacts of a proposed marina development in a harbour site (salinity 20–32‰) where there are contaminated sediments that need to be dredged and disposed of nearby. The assessment requires consideration of potential impacts of:

(i) disturbing the sediment at the existing location (for example, by installing piles into sediment for wharfs); and
(ii) contaminants in the sediments that are being considered for dredging and relocation, adding toxicity at the disposal site.

A toxicity assessment should be included in the full ecological risk assessment. That assessment should consider not only sediment toxicity but also other aspects of the chemistry and ecotoxicology lines of evidence (see Chapter 1), such as the release and potential effects of any contaminants within the water column, both during disturbance at the site, and during sediment dredging and disposal. For example, the water-column assessment might use elutriate tests on representative sediments to assess the potential for contaminant release due to dredging, in comparison with water quality guideline (WQG) values, taking into account possible dilution (see Chapter 2 Section 2.13). This might indicate a need to avoid or minimise disturbing some sediments, or determine a need for additional water quality monitoring. The potential for bioaccumulation (Chapter 5) and ecological impacts (Chapter 7) should also be considered.

Sediment properties

The sediments comprise mostly silty sand (60–95% <63 µm) to a depth of 0.5–1 m, overlying progressively more clayey materials, with hard clay beyond 2.5 m depth. They have received contaminants over many years from agricultural and urban run-off and inputs from a range of existing and former industries. All of the sediments being considered are sub-tidal and the location has a high rate of water exchange from tides. There are no seagrasses, but there is evidence of bioturbating organisms such as polychaetes and possible large bivalves.

Sediment contaminants

The contaminants of potential concern (COPCs) are determined as being those for which the 95% upper confidence limit (95% UCL) exceeds the sediment quality guideline values (SQGVs, Appendix A) or where total concentrations exceed the SQGV-high values in any individual sediment samples. The 95% UCLs for the metals Cr, Cu, Ni, Pb and Zn (50–820 mg/kg range) exceed the SQGVs (with individual samples exceeding SQGVs by up to a factor of 4). Approximately 30% of the sediment samples have Cu, Pb or Zn concentrations greater than the SQGV-high values (95% UCLs < SQGV-high). No other contaminants have 95% UCLs exceeding SQGVs, although exceedances for individual sediment samples are observed for polycyclic aromatic hydrocarbons (PAHs) in 10% of samples (in the range from <0.2 to 18 mg total PAHs/kg (normalised to 1% total organic carbon)), total petroleum hydrocarbons (TPHs) in 8% of samples (100–600 mg TPHs/kg range) and tributyltin (TBT) (occasional samples with 5–80 μg Sn/kg). The deeper sediments are considerably more contaminated than the surface sediments (that is, there are more samples there that exceed the SQGVs), probably owing to improved discharge regulation, and because there is less contamination in the fine sediments that have more recently been deposited over the historical contaminants from the more industrial era. Concentrations of organochlorine and organophosphate pesticides and polychlorinated biphenyls (PCBs) are determined to be negligible, and emerging contaminants are not expected to be an issue based on preliminary assessments (historical data). Thus Cr, Cu, Ni, Pb and Zn are classified as the COPCs.

Chemistry lines of evidence

Additional sampling and analyses provide information on the potential bioavailability (see Chapter 3) of the major COPCs.

- Dilute-acid extractable metal (AEM, Chapter 3 Section 3.5.1) analyses determine that 60–80% of the total metal concentrations are present in AEM forms (that is, forms that may be potentially bioavailable). The AEM concentrations of chromium and nickel are below the SQGVs for all samples. The 95% UCLs of AEM Cu, Pb and Zn concentrations exceed the SQGVs (with individual samples exceeding SQGVs by up to a factor of 3). Approximately 20% of the sediment samples have AEM concentrations of zinc exceeding the SQGV-high values. The metals Cu, Pb and Zn are therefore identified as contaminants of concern (COC).
- The bioavailability of metals is likely to be quite different in the surface sediments from the deeper sediments, as there is clear stratification of redox layers: a 0–5 mm pale brown surface layer (oxic), followed by a 5–25 mm depth layer that becomes progressively greyer and darker (sub-oxic), becoming dark grey to black coloured (with mild sulfide smell) down to the clay layer.
- Analyses of filtered pore water (following separation by centrifugation under nitrogen; see Chapter 2 Section 2.11.1) and AVS (see Chapter 3 Section 3.6.1 and Appendix C) indicate an excess of AVS that should decrease the bioavailability of the Cu, Pb and Zn in the deeper sediments. Potential oxidation and increased metal bioavailability are likely if the disturbance or relocation of deeper sediments results in their exposure to oxygenated waters.
- Occasionally the surface sediments, but not the deeper sediments, have TBT concentrations that exceed the sediment quality guideline values (possibly from paint flakes), while detectable PAHs and TPHs only exist in the surface sediments.

Ecotoxicology lines of evidence

To ensure a sound ecotoxicological assessment, care is taken to develop a robust experimental design to demonstrate that the desired exposure has been achieved, along with appropriate controls and endpoints, including an unbiased analysis of the results (see Section 4.1).

Along with a preference for sub-lethal endpoints that provide chronic measures and therefore provide greater information on potential for long-term effects at the individual and population levels, careful consideration is given to the selection of organisms (see Section 4.4).

Choice of bioassay

There is a need to use organisms that live in close association with sediments. Two very robust and well established sub-lethal but relatively rapid (10-day) whole-sediment bioassays are selected: 10-day survival and reproduction of the amphipod *Melita plumulosa* (acute and chronic endpoints) and 10-day reproduction of the harpacticoid copepod *Nitocra spinipes* (chronic endpoint) (see Table 4.1 and Appendices E and F). These species have different exposure routes, they are relatively sensitive to metals, and their reproduction is not adversely affected by physical sediment properties (that is, there are good control responses in both sand and silt).

For a third bioassay, the question is whether to include a further whole-sediment test without a chronic endpoint; for example, 24-h inhibition of esterase activity in a benthic alga species, or 10-day survival of a benthic bivalve (both classified as acute toxicity), or a chronic pore-water bioassay. The available acute bioassays may not be adequately sensitive (that is, may potentially assess sediments as being non-toxic when in reality they can cause chronic effects), while pore-water tests do not capture all exposure pathways and may suffer several artefacts (for example, once isolated, pore waters are unstable and the dissolved concentrations of COPCs decline with time, possibly because of, for example, precipitation of metals or volatilisation of organics; see Section 4.3.1). The decision is made to use a sub-lethal pore-water test; namely, the 72-h larval development of the sea urchin *Heliocidaris tuberculata* as it is more sensitive to dissolved contaminants than other available whole-sediment tests.

During pore-water testing, daily measurements are made of dissolved ammonia, to which the larvae are very sensitive, and also of dissolved metals, to provide information on the rate of decrease in any dissolved COPCs. In the presence of sediments, the concentrations of dissolved COPCs would be expected to be maintained through equilibrium with the particulate phase; but in a pore-water exposure the concentration is likely to decrease (often by >20% within an hour, and then to a greater extent over days).

If only one relevant chronic whole-sediment test was available, then further acute whole-sediment tests and/or further toxicity tests on pore waters would be included, with acknowledgement of their potential deficiencies (for example, lower sensitivity or incomplete exposure assessment). If dioxins/EDCs were major COPCs, then some of the cell-line bioassays that are specific to effects from these classes of toxicants (for example, AhR-CAFLUX targets dioxins and ER-CALUX® targets EDCs; see Section 4.9) would be recommended.

Choice of sediments

Bioassays are undertaken on the more contaminated sediments that represent the worst case scenario for toxicity (for example, representing the 95% UCL concentration, or greater, for each COPC). The observation of no toxic effects for these sediments will provide

greater confidence that the final assessment decision is adequately conservative. The sediments and COPCs considered for cases (i) and (ii) (above) are different, with the latter considering a greater portion of the deeper sediments that have generally higher metal concentrations, to provide a representative assessment in each case.

A new campaign of sediment sampling is undertaken to provide sufficient material (for example, ~3 kg per location) for consideration both of chemistry and ecotoxicology lines of evidence. Total COPC concentrations are determined and four representative samples are collected for toxicity testing in assessment case (i) and eight for assessment case (ii). The number of samples tested is based on the assessment areas and volumes (see NAGD, 2009). The sampling and analysis plan directs that ecotoxicology assessment should commence within three weeks of sample collection (with samples stored under refrigeration in plastic bags with minimal air exposure) to reduce the potential changes in contaminant concentrations and bioavailability that may occur during storage (DeFoe and Ankley GT, 1998; Simpson and Batley, 2003).

Results of toxicity testing

The test acceptability criteria (see Section 4.7.4) are met for both assessments.

Toxicity assessment for case (i): Disturbing the sediment at the existing location

- No acute toxicity is observed in any sediment using any of the whole-sediment toxicity tests (that is, there is >80% survival of adult organisms). Sub-lethal effects to amphipod and copepod reproduction are observed for one of the four sediments tested (60–75% of reproductive output observed for control and reference sediments), but in different sediments for each organism. These two sediments do not have particularly high concentrations of COPCs or any other measured chemical features that are outstanding, and both species endpoints are not affected by non-chemical factors such as particle size differences of the assessed sediments.
- Toxicity is observed in two of the six pore-water toxicity tests (30–40% effects to larval development), but these include only one of the sediments for which sub-lethal effects are observed (to the amphipod). However, dissolved ammonia analyses of pore water and test waters on Day 3 indicate that ammonia (4–10 mg/L) is likely to be the major cause of the observed toxicity (that is, above the EC50 for the sea urchin larvae development endpoint). The ammonia toxicity is not considered to be a major assessment issue because the pore-water concentrations are within the range found elsewhere in the harbour sediments and concentrations in the overlying water are low (chemistry lines of evidence assessment). Furthermore, it is recognised that many benthic organisms are either tolerant of, or have mechanisms for dealing with, elevated pore-water ammonia, whereas the sea urchin and its larvae will not be directly exposed to pore water naturally.

Overall outcome for assessment case (i)

The sediments represent a **low risk of adverse effects to benthic organisms**. Disturbing the sediments by boats (for example, turbulence from propellers) or by installing piles into sediment for wharfs is unlikely to increase the risk of adverse effects to benthic organisms posed by the sediments.

Toxicity assessment for case (ii): Sediments that are being considered for dredging and relocation

- For the amphipod, acute lethality (50–60% survival) and no reproduction are observed in two of the eight sediments tested (a different range of sediments from

those tested in assessment case (i)). While these sediments have highly elevated concentrations of COPCs, they are also quite compact clays in which the amphipods have difficulty burrowing. Dissolved metal analyses made throughout the tests determine that the copper and zinc concentrations exceed the EC50 for reproduction and the EC10 for survival of the amphipod.

- Sub-lethal effects to reproduction are observed in six of the eight sediments tested for the amphipod (35–70% effects) and in four of the eight sediments for the copepod (40–65% effects).
- All of the sediments that cause sub-lethal toxicity to the copepod reproduction also cause toxicity to the amphipod reproduction (one has a high clay concentration that also might be responsible for acute toxicity), and there is a relationship indicating increased toxicity with increased concentrations of dilute-acid extractable metals (Cu, Pb and Zn) (although the sample size is not large enough to statistically analyse potential dose–response relationships). No detects are reported for organic contaminants or TBT in these deeper sediments, and these classes of contaminants are largely discounted as potential toxicants in the tested sediments.
- Toxicity is observed in two of the eight pore-water toxicity tests (35–45% effects), and again this is attributed to pore-water ammonia (see assessment case (i) discussion).

Overall outcome for assessment case (ii)

While there are often challenges with undertaking toxicity tests on hard clay materials when physical factors are likely to have contributed to acute toxicity observed in the amphipod bioassays, the high frequency of effects to both amphipod and copepod reproduction result in the finding that the deeper sediments represent a **potential risk of adverse effects to benthic organisms.** These sediments may cause adverse effects at the site of disposal.

Assessment summary

In combination with the chemistry line of evidence assessment of high concentrations of potentially bioavailable metals in the deeper sediments, the ecotoxicology line of evidence indicates that, overall, the sediments would be unsuitable for dredging and disposal at a location that has similar ecological values. For the identified COPCs (Cu, Pb and Zn), the assessment of bioaccumulation by benthic or pelagic organisms would not be expected to improve or modify the assessment outcome. Dredging and disposal elsewhere or on land may be an option, but additional assessment may be necessary to determine whether any deeper sediments that remain following dredging pose ongoing risks to the ecosystem (natural sedimentation may be sufficient to result in remediation; that is, burial within months to years and recolonisation by benthic communities).

Alternative options to dredging and disposal may also exist for marina development. They include:

(i) capping of the undesirable sediments at a disposal site with a clean material to prevent organisms from interacting with materials that have been assessed as posing potential risk of adverse effects, and

(ii) undertaking a more detailed assessment of the contaminant concentrations, bioavailability and ecotoxicology, to develop potential effect relationships for the major contaminants of concern (COC). These relationships can be used to identify thresholds suitable for deriving scientifically defensible site-specific management limits for classifying sediments as suitable and unsuitable, for dredging and unconfined disposal or for retaining uncapped.

4.12.2 Freshwater lake assessment case study

The issue

A freshwater lake close to, and sometimes inter-connected to, a large river system, receives some effluent runoff from dairy farming (potential inputs of antibiotics and natural hormones through excreta and faecal runoff into drains), mixed agriculture (various pesticides) and a small urban population. All sources potentially contribute excessive nutrients. When water levels are high within the river system (particularly during floods), river water flows back into the lake. Upstream (~3 km), the river receives input from a sewage treatment plant (STP, secondary treatment) and the environment surrounding the river has intense agriculture and animal farming (cows, sheep) which extract and return water to the river system.

The lake supports a moderate recreational fishery and is also of ecological significance. The local residents use the lake for recreation (sailing and swimming) and are concerned about the degradation of the environment following intensification of land use. Concerns have been raised specifically about the health of the sediments and an ecotoxicology assessment has been requested as part of a wider sediment quality assessment.

Sediment properties

The sediments comprise silty sand (75–95% <63 μm) to a depth of 1 m, overlying hard clay and bedrock. They have received contamination over many years from agricultural run-off (including dairy inputs) and urban run-off and from the sewage treatment plant outfall and from a range of existing and former industries. There is evidence of bioturbating organisms such as European carp (*Cyprinus carpio*), polychaetes and possible large freshwater mussels (*Velesunio ambiguus*).

The use of three lines of evidence – chemistry, toxicity and macroinvertebrate community assessment (Chapter 1 Section 1.4) – is recommended, on sediment sampled at 15 locations within the river and lake system. The combination of these measurements of potential cause (chemistry) and effect (biology) provides a powerful tool when investigating sediment pollution and potential impacts, which will be used to address each of the four guiding questions:

1. Are contaminants generated in the system?
2. Are contaminants bioavailable?
3. Is there a measurable response?
4. Are the contaminants causing this response?

Sediment contaminants

The contaminants of potential concern (COPCs) are mainly metals (Cu, Pb and Zn) in the 100–800 mg/kg range, typically 2–4 × guideline values. Pore-water chemistry also indicates that dissolved Cu, Pb, Zn and Ni are above the water quality guideline values. Concentrations of organochlorine pesticides and polychlorinated biphenyls (PCBs) are determined to be negligible. Organophosphate pesticides such as chlorpyrifos, and pyrethroids such as cypermethrin and bifenthrin, and herbicides such as simazine are detected at μg/kg levels. Nonylphenol, octylphenol and carbamazepine are detected in sediments at low μg/kg concentrations. Currently, sediment quality guideline values (SQGVs) are not available for these emerging contaminants, which are not expected to be an issue based on preliminary assessments (historical data).

Chemistry lines of evidence

Sediment sampling is conducted at 15 locations within the river and lake system, and analyses of the major COPCs (see Chapter 3) provide the following information:

- Dilute-acid extractable metal (AEM, Section 3.6.1) analyses determine that 70–85% of the total metal concentration is present in AEM forms (that is, forms that may be potentially bioavailable). The AEM concentrations of Cu, Ni and Pb are below the SQGVs for all sediment samples. The 95% UCLs of AEM Zn concentrations exceed the SQGVs (with individual samples exceeding SQGVs by up to a factor of 3–5). In addition, chromium and arsenic are also found to be above the guideline levels (with individual samples exceeding SQGVs by up to a factor of 2–3) at four locations. Approximately 20% of the sediment samples have AEM concentrations of zinc and arsenic exceeding the SQGV-high values. The metals Zn, Cr and As are therefore identified as contaminants of concern (COC).
- Analyses of filtered pore water (following separation under nitrogen by centrifugation, see Chapter 2 Section 2.11.1) and AVS (see Chapter 3 Section 3.6.1) indicate an excess of AVS that should decrease the bioavailability of zinc and ensure that chromium is present as Cr(III) in the deeper sediments at 11 sites sampled. Potential oxidation of AVS and increased metal bioavailability are likely if the disturbance or relocation of deeper sediments results in exposure to oxygenated waters.
- Total petroleum hydrocarbons are detected in the surface sediments of 13 sites. Organochlorine pesticides such as DDE and dieldrin are found in the deeper layers (below 5 cm depth) of sediments from all sites, confirming historical contamination; DDT was banned in the 1980s. Chlorpyrifos, cypermethrin, deltamethrin, simazine, atrazine and imidacloprid are detected at 0.05–0.45 µg/kg concentrations at seven of the sites selected in this investigation. Traces of two antibiotics are detected at two sites but are close to their detection limits.
- Nonylphenol and octylphenol are detected at all sites at low µg/kg concentrations. Carbamazepine is detected in sediments at four sites and the concentrations vary from 0.04 µg/kg to 0.120 µg/kg.

Macroinvertebrate community assessment

Documentation of the structure of benthic macroinvertebrate communities through the taxonomic identification and enumeration of field-collected organisms is also used to assess sediment quality. Benthic macroinvertebrates are relatively sedentary organisms that inhabit or depend on the sedimentary environment for their various life functions. Therefore, they may be sensitive to both long-term and short-term changes in habitat, sediment, and water quality. Sediment core samples are collected to a depth of 10 cm and divided into three layers: 0–2 cm, 2–5 cm and 5–10 cm. Four replicate samples from each of 15 sites are processed individually by washing each sample through a 250 µm sieve and enumerating and identifying all macroinvertebrates in the samples.

The following parameters are calculated for each site: taxa richness, total abundance, proportion of oligochaetes and the Benthic Index of Biotic integrity (B-IBI) based on the Weisberg *et al.* (1997) method. B-IBI scores range from 1 to 5. Sites with scores greater than or equal to 5 are considered to be undegraded. Sites with scores 2.7–2.9 are marginally degraded; with scores 2.1–2.6 are degraded; and scores of 2 or less indicate a severely degraded site (Llansó *et al.*, 2003).

Results of benthic community analysis

Based on macroinvertebrate sampling and assessment, the following observations are made:

- A total of 33 taxa are identified from the 15 sites in the river and lake system.
- All sites are dominated by oligochaetes which range from 45% of the organisms at one of the river sites to 95% at a sewage-impacted site. Other taxonomic groups include midges, leeches, molluscs, crustaceans and other insects.
- Taxa richness ranges from 35 at the second reference site to 5 at the sewage-impacted site.
- B-IBI indicates three of the 15 sites are classified as severely degraded, six are marginally degraded and the other six are not degraded.

Ecotoxicology lines of evidence

To ensure a sound ecotoxicological assessment, care is taken to develop a robust experimental design to demonstrate that the desired exposure is achieved, along with appropriate controls and endpoints, including an unbiased analysis of the results (see Section 4.2). Additionally, there is a preference for sub-lethal endpoints that provide chronic measures and therefore provide greater information on potential for long-term effects at the individual and population levels. Careful consideration is given to the selection of organisms (see Section 4.4).

A new campaign of sediment sampling is undertaken to provide sufficient material for both chemistry and ecotoxicology line of evidence considerations (for example, ~3 kg per location). Total concentrations of COPCs are determined and four representative samples are collected for toxicity testing assessment, where this number of samples is selected based on the assessment area and the use of a targeted sampling approach. The sampling and analysis plan directs that ecotoxicology assessment should commence within 3 weeks of collection (with samples stored under refrigeration in plastic bags with minimal air exposure) to reduce potential changes in contaminant concentrations and bioavailability that may occur during storage.

Approaches for ecotoxicological assessment

Laboratory-based bioassays (both *in vitro* and *in vivo*), *in situ* caging studies and field studies all can be included for the integrated ecotoxicological assessment. The following approaches are used in this investigation.

- Recognising the need to use organisms that live in close association with the sediments, one very robust and well established sub-lethal but relatively rapid (10-day) whole-sediment bioassay is selected: 10-day survival and reproduction of midge larvae (*Chironomus tepperi*, acute and chronic endpoints). For detailed information see Table 4.1 and Appendix H). Midge larvae are relatively sensitive to metals and organic contaminants and their reproduction is not adversely affected by physical sediment properties (that is, good control responses are possible in both sand and silt) or by conditions with variable pH (as low as 5) and electrical conductivity (250–4000 µS/cm).
- Toxicity identification evaluation (TIE) phase 1 manipulations are used to identify the contaminants contributing to the toxicity observed (see Section 4.10 above). To evaluate toxicity due to cationic metals, the sediments are treated with a 10% SIR-300 (ResinTech, West Berlin, NJ, USA), which is a macroporous weak-acid cation-exchange resin that has chelating properties for metal ions and is used to reduce bioavailability of cationic metals. For measuring the toxicity of organic contaminants, sediments are treated with 10% pyrolysed activated coconut husk (PCC), ground to

<45 μm (90–96%, Calgon Carbon, Pittsburgh, PA, USA), which is added to sediment to reduce the bioavailability of organic contaminants. Midge bioassays are run and the results are compared to the baseline toxicity.

- As only one relevant chronic whole-sediment test is available, further toxicity tests use pore waters, with acknowledgement of their potential deficiencies (for example, lower sensitivity or incomplete exposure assessment; isolated pore waters are unstable and the dissolved concentrations of metals or organics may decline with time; Section 4.3.1). The selected tests are a 48-h water flea *Ceriodaphnia dubia* acute bioassay and a 7-day sub-lethal pore-water test using fish embryos from native species such as golden perch, purple spotted gudgeon or Murray rainbowfish. The latter test will assess fish larval survival and growth. Unfiltered pore waters are used.
- During pore-water testing, daily measurements are made of dissolved ammonia, to which the water fleas and fish larvae are very sensitive, and also of dissolved metals (and organics where feasible) to provide information on the rate of decrease in any dissolved COPCs. In the presence of sediments, the concentrations of dissolved COPCs would be expected to be maintained through equilibrium with the particulate phase.
- Dairy and sewage effluent inputs into this river and lake system could lead to endocrine disruption in the organisms inhabiting these waters. Therefore a suite of *in vitro* screening tests is used to assess the contribution of micropollutants such as dioxins or EDCs as major COPCs, using the cell-line bioassays as described in Section 4.9.
- *In situ* assessments of toxicity and the bioaccumulation of contaminants are carried out using the freshwater yabby *Cherax destructor* and the snail *Physa acuta* (using the protocol described in Appendix I).

Results of toxicity testing

The test acceptability criteria (see Section 4.7.4) are met for the ecotoxicological assessments. The results are summarised below:

- No field (lake) sediments are acutely toxic to *C. tepperi,* with mean survival greater than 80% in all samples, and no significant differences between any lake sites compared to the external reference site sediment (control). However, there are significant differences in *C. tepperi* growth when exposed to sediments from six sites within this river and lake system. Midge emergence is significantly delayed when exposed to sediments from four sites. Sediments from other locations do not affect the midge emergence.
- For the TIE manipulations, both SIR-300 and PCC treatments increase midge larval growth by more than 25% compared to no manipulation at six sites, suggesting there are effects both of metals (considered most likely to be due to zinc) and of organic compounds on the growth of *C. tepperi* within the river and lake system.
- Pore-water toxicity is observed for eight of the 15 sites within the river and lake system. The effects are highly variable and toxicity is removed with >50% dilutions of the pore-water samples from these eight sites.
- Estrogenic and (anti)androgenic activity (up to 5 ng/L estradiol equivalent) are detected in the sediment extracts from sites near to the STP outfall, but they are not detected at all 1 km downstream. In the receiving environment, glucocorticoid and progestogenic activity are also detected up to 4 km downstream of the effluent outfall. Sediments from the dairy site exhibit estrogenic activity up to 10 ng/L, but this is completely absent in the sediment extract collected 2 km downstream.

- Dissolved oxygen, pH and temperature are similar between sites, while electrical conductivity is higher at STP outfall site relative to all other sites. Freshwater snail survival is similar between the laboratory reference and the field reference sites. Survival of caged snails decreases during 7-day exposures at seven sites. There is high variability in snail survival between cages across all sites with maximum mortality at the mixing zone closer to the sewage outfall and the site receiving dairy effluent.
- Yabbies translocated at the sites closer to the STP outfall and the dairy accumulate simazine, chlorpyrifos and the metals/metalloids As, Cr and Zn during the 8-week exposure period.

Weight-of-evidence conclusions

All lines of evidence (chemistry, toxicity, macroinvertebrate diversity, and bioanalytical responses) indicate that the two reference sites are not contaminated and display no evidence of adverse effects. Sites below the sewage outfall and the site receiving dairy wastewater are severely impacted, with high concentrations of a suite of inorganic and organic contaminants, and clear adverse effects occurring to the macroinvertebrate community (low B-IBI). TIE manipulations indicate that both metals/metalloids and organics are responsible for the sub-lethal toxicity in *C. tepperi*. Sites that are 4 km downstream of the STP and dairy farm, overall, support healthy invertebrate populations.

Several management options can be suggested for this river and lake system.

- Effluent from the STP could be given tertiary treatment before being released into the river. In addition, zero discharge into the river, and exploring irrigation schemes to reuse this nutrient-rich water, would add value.
- The dairy farmers could also withhold their wastewaters and construct dams to prevent run-off into the river.
- Pesticide use could be reduced by developing integrated pest management strategies and educating farmers on the safe use of pesticides. Buffer strips along the river bank and lake would further reduce the runoff.
- Undertaking a more detailed assessment of the contaminant concentrations, bioavailability and ecotoxicology could potentially develop effect relationships for the major contaminants of concern (COC). These could then be used to identify thresholds suitable for deriving scientifically defensible site-specific management.

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5

Bioaccumulation

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5.1 Introduction

Bioaccumulation refers to the accumulation of contaminants in the tissues of organisms through any route, including respiration, ingestion, or direct contact with contaminated sediment or water (USEPA, 2000a; Rainbow, 2007; ASTM, 2010). Organisms living in or on sediments are able to bioaccumulate contaminants from both pore waters and overlying waters and via ingestion of sediment particles and food. Organisms suitable for use as 'biomonitors' have the capacity to concentrate those portions of contaminants that are in a bioavailable form.

Metals and metalloids bioaccumulate particularly in organisms at lower trophic levels, such as some polychaetes and molluscs (Taylor and Maher, 2003; Waring *et al*., 2006), and trophic transfer may be observed; for example, transfer of metals from phytoplankton to filter-feeding molluscs and herbivorous gastropods or barnacles and then to carnivorous gastropods and polychaetes (Wallace *et al*., 1998; Zhang and Wang, 2006; Rainbow and Smith, 2010). Generally, biomagnification (increase in concentration through three or more trophic levels) does not occur with metals (Goodyear and McNeill, 1999; Cardwell *et al*., 2013) but can occur for the metalloid selenium and mercury (Bowles *et al*., 2001; Barwick and Maher, 2003).

Most non-ionic hydrophobic organic chemicals (HOCs), such as PCBs and PAHs, are readily taken up by organisms and accumulate in tissues (Moore *et al*., 2005; ASTM, 2010). Synthetic organic chemicals such as PCBs are highly resistant to metabolic degradation and so can accumulate to high concentrations (Fiedler *et al*., 1994). Some organic chemicals, for example PAHs, are readily taken up by many organisms but are rapidly metabolised (Maher and Aislabie, 1992). Many non-ionic organic chemicals are lipophilic so can also biomagnify through food chains (Kelly *et al*., 2007).

A comparison of bioaccumulated concentrations in sedentary organisms from different sites can assist in assessing the risk posed by contaminants and sources. The organisms can be either field-sampled or caged. Phillips (1980) has identified three categories of factors that may contribute to changes in tissue contaminant concentrations through time:

- variation in contaminant delivery to the environment;
- changes in ambient factors affecting metabolism, such as salinity and temperature; and
- the organisms' physiology, especially aspects relating to reproductive cycles and changes in mass.

These factors rarely operate in isolation, and the interactions between them complicate interpretation of their combined effects.

5.1.1 Bioaccumulation as a line of evidence

The focus of this chapter is on the use of bioaccumulation data as a line of evidence to indicate that organisms have been exposed to bioavailable contaminants. That evidence is valuable support for other lines of evidence, such as measures of high concentrations of sediment contaminants (Chapter 3), or toxicity (Chapter 4), or biomarker responses (Chapter 6). Attempting to quantify bioaccumulation to provide a ranking is somewhat arbitrary, the intent being to derive a ranking of accumulated tissue concentrations from values measured to represent ranges for unimpacted, potentially impacted and impacted. A ranking scheme recommended for Australia and New Zealand (Chapter 1, Section 1.4) has three classes: (i) Not significantly different from control; (ii) Significantly different ($P < 0.05$) and $\leq 3 \times$ control; and (iii) Significantly different ($P < 0.05$) and $>3 \times$ control. These rankings are, however, arbitrary and meaningless unless the baseline contaminant concentrations and potential toxicity are taken into account. Species or types of organisms will have different natural baseline concentrations of essential metals such as copper and zinc, and changes in concentrations relative to baseline will have different effects in different organisms. Selenium is an example of an element with a narrow concentration range: there is little difference between it being essential and being toxic, and small increases can have deleterious effects (Janz *et al*., 2010). In the case of HOCs, the baseline concentrations should be zero.

As the following pages will show, detailed investigations can improve the value of bioaccumulation assessments by providing an overview of sources of contaminant variability in organisms, and knowledge of the range of concentrations that may found in clean natural background situations. Bioaccumulation beyond that range then may be an indication of contaminated sediment.

5.2 Use of bioaccumulation data

5.2.1 Measures of bioaccumulation

Data for use in a weight-of-evidence (WOE) assessment are best obtained from measurements of contaminant concentrations either in field-collected native organisms or in field-transplanted (caged) organisms (which may be sourced from aquaculture). Organisms can be exposed to collected sediments in the laboratory, but diffusion of contaminants from pore water can result in elevated contaminant concentrations in the overlying water which are not representative of a field situation, even when frequent changes of overlying water are made.

The simplest assessment involves measuring contaminant bioaccumulation from a particular sediment (at a test location or in a laboratory test) and comparing that to the bioaccumulation of the same contaminant from at least three reference sediments, to establish whether a statistically significant difference exists.

Alternatively, if background concentrations of contaminants within organisms are known, these can be used to determine the bioaccumulation ratio. For example, in Australia, an extensive database has been established of metal concentrations in the oyster *Saccostrea glomerata* that inhabits the NSW–Queensland coast (Scanes and Roach, 1999; Robinson *et al*., 2005).

Bioaccumulation can be modelled (USEPA, 2000b). However, in the context of a weight-of-evidence assessment, modelling is generally considered inappropriate beyond use in

screening to determine which contaminants should be included in the assessment. Simple equilibrium partitioning models have been useful for predicting the bioaccumulation of HOCs from sediments (for example, Di Toro and McGrath, 2000). Biodynamic models that consider the uptake and efflux rates of metals from water and dietary routes have been used for predicting bioaccumulation of some metals (Luoma and Rainbow, 2005). For accurate predictions, a strong knowledge of geochemical and biological influences on metal bioavailability is needed, including the effects of feeding strategies on exposure (Simpson, 2005; Baumann and Fisher, 2011a,b; Höss *et al*., 2011; Camusso *et al*., 2012; Yu *et al*., 2012; Proulx and Hare, 2014; Campana *et al*., 2015).

5.2.2 Prediction of effects

Once bioaccumulation has been established, the significance of the accumulated concentrations both to ecosystem health and to human health may need to be determined. Where bioaccumulated concentrations of a contaminant exceed maximum residue limits in organisms for human consumption (FSANZ, 2013), the first management action might be to ban the collection of the affected species, at the same time evaluating remediation options to remove the contaminant source by approaches such as dredging or capping. Assessing the risk to ecosystem health is more problematic. High concentrations of a bioaccumulated contaminant are not necessarily linked to toxicity in an organism, and more detailed investigations will be needed to assess if toxicity is occurring. These investigations would involve other lines of evidence such as toxicity testing (Chapter 4) or the use of biomarkers (Chapter 6) to determine the extent to which the organism's biological functions are altered or impaired as a result of the bioaccumulation.

Organic contaminants

For HOCs, bioaccumulation can be directly related to toxicity and used as a valuable tool for assessing the effects of mixtures of HOCs and for developing guidelines (Meador *et al*., 2011; Burgess *et al*., 2013) (see also Chapter 3, Section 3.6). The bioaccumulation of HOCs is dependent on many factors, including exposure medium, uptake rate, metabolic capability, lipid content, and feeding strategy (Meador *et al*., 1995; Moore *et al*., 2005; Meador, 2006).

Two factors, lipid and organic carbon content, control to a large extent the partitioning behaviour of non-ionic organic chemicals between sediment, pore water and tissue (Ankley *et al*., 1992; USEPA, 2000b). These two factors, along with the octanol:water partition coefficient (K_{OW}), have been used in simple equilibrium partitioning (EqP) models to predict the partitioning and bioaccumulation behaviour of PAHs in sediments (Di Toro *et al*., 1991, 2000; Meylan *et al*., 1999; Di Toro and McGrath, 2000).

Biota-to-sediment accumulation factors (BSAFs) and theoretical bioaccumulation potential (TBP) are screening tools based on equilibrium partitioning models which are useful for estimating the bioaccumulation of persistent non-ionic organic chemicals by benthic organisms exposed to contaminated sediments (USEPA/USACE, 1998; USEPA, 2000a; Moore *et al*., 2005). For organics, BSAFs are typically derived using a sediment concentration (C_S) normalised to organic carbon (f_{OC}) and a tissue concentration normalised to its lipid content (f_L) (Moore *et al*., 2005). The TBP is the expected concentration in an exposed organism's tissues and is the simplest and most easily understood model for estimating bioaccumulation, but it is also subject to a large degree of uncertainty (USEPA/USACE, 1998; USEPA, 2000a). It is related to BSAF by the equation:

$$TBP = BSAF \times (C_S / f_{OC}) \times f_L.$$

For many HOCs, equilibrium partitioning theory provides useful relationships between water concentrations, bioaccumulation and toxic effects to some benthic organisms, although the approaches may under- or over-estimate bioaccumulation (Di Toro and McGrath, 2000; McGrath and Di Toro, 2009). The quality of predictions of TBP is therefore dependent on the choice of BSAF, and a BSAF–lipid database is available (USACE, 2014). The models do not consider the kinetics of processes that determine contaminant bioavailability from sediments, nor contaminant retention, metabolic degradation, or elimination from organisms. Equilibrium partitioning approaches do not adequately consider sediment ingestion by marine invertebrates as a major exposure pathway (Meador *et al*., 1995; Kaag *et al*., 1997; Baumard *et al*., 1999). For deposit-feeding bivalves and worms particularly, sediment ingestion is a major uptake route (Kaag *et al*., 1997; Mackay and Fraser, 2000; Weston and Maruya, 2002; Meador, 2006; Maruya *et al*., 2012; Burgess *et al*., 2013). The equilibrium partitioning approach usually does not take into account the different forms of organic carbon present in sediments. Desorption kinetics of HOCs vary greatly depending on organic and sediment characteristics (Cornelissen *et al*., 1997; Hendriks *et al*., 2001; Kraaij *et al*., 2003). McGroddy *et al*. (1995) found only a fraction (0.01–0.4, or 1–40%) of sediment-associated PAHs appears to be involved in equilibrium partitioning with the pore water. Thus, adsorption and desorption kinetics, which are not considered by equilibrium partitioning approaches for estimating bioaccumulation, may greatly affect partitioning and bioaccumulation.

As described in Chapter 3, Section 3.6, relationships between sediment concentrations, tissue concentrations and toxic effects have been used to develop mechanistic guidelines for many HOCs (Di Toro and McGrath, 2000; McGrath and Di Toro, 2009). An environmental residue-effects database (ERED) has also been developed for studies where both tissue contaminant concentrations and biological effects have been measured (USACE/USEPA 2015). The toxico-kinetics and toxico-dynamics of bioaccumulated chemicals also play a large role in determining if and when effects may occur, and to what magnitude (McCarty *et al*., 2011; Ashauer and Brown, 2013).

Metal contaminants

Most of the metals taken up by an organism do not bioaccumulate, but instead are processed internally and excreted (Wallace *et al*., 2003), with only a fraction of the metals remaining in forms that contribute to toxicity within the organism (Vijver *et al*., 2004; Luoma and Rainbow, 2005; Rainbow, 2007). For most metals, this means that bioaccumulation data cannot be used to predict the risk of toxicity to an organism (Adams *et al*., 2011). Oysters, for example, can accumulate high concentrations of copper and zinc, but still function (Pan and Wang, 2012), and it has been demonstrated that oysters (and other bivalves) are able to sequester metals into sub-cellular non-toxic forms with only very small amounts of metals remaining in metal-sensitive cell components (Wang *et al*., 2011).

Biota-to-sediment accumulation factors (BSAFs) were never intended for use with metals (Moore *et al*., 2005). Literature generally indicates that BSAF data for metals show extreme variability. Hence it is inappropriate to use BSAFs as criteria for identifying and classifying metals as hazards.

To use metal bioaccumulation data for the assessment of toxic effects, it is necessary to determine the tissue metal concentration that will cause adverse effects. The critical body residue (CBR) approach has been used to model dose–response relationships in aquatic organisms (USACE/USEPA, 2002). The strengths of the CBR approach are that

bioavailability, exposure to food, and accumulation/depuration rate kinetics are explicitly addressed. The major uncertainty of the approach is in determining a dose or response that is protective of ecological health (McGeer *et al*., 2003). Metals bind at a range of sites within organism tissues that have different functions, and impairment of function is the potential cause of toxicity within an organism (Wallace *et al*., 2003; Vijver *et al*., 2004; Rainbow, 2007). Uncertainties in using the CBR approach have been discussed by Moore *et al*. (2005) and Adams *et al*. (2011).

Organisms' body concentrations of metals may provide useful predictions of possible effects only if strong and clear relationships exist between bioaccumulation and biological effects (Borgmann, 2000; Borgmann *et al*., 2004; Simpson and King, 2005; Rainbow, 2007; Adams *et al*., 2011). Borgmann *et al*. (1991, 1998, 2001) found that chronic toxicity of Cd, Tl and Ni to the freshwater amphipod *Hyalella azteca* was a function of the total amount of metal accumulated, and not the total metal concentration in water or sediment. These relationships were used to calculate lethal body concentrations (LBCs) and internal effect concentrations (IECs). Taylor and Maher (2010, 2012a,b,c, 2013, 2014b) have shown that there are clear significant relationships between metal accumulation (Cd, Pb and Se) in the bivalves *Anadara trapezia* and *Tellina deltoidalis* and sub-lethal effects (antioxidant capacity, lipid peroxidation and lysosomal destabilisation). Marasinghe Wadige *et al*. (2014a,b) have shown similar relationships for the freshwater bivalve *Hyridella australis*. These data can also be used to define metal concentrations at which harmful effects can occur. For metals that are sequestered in non-toxic forms or are regulated over the concentration range of interest, the use of body concentrations to predict effects is not appropriate (Borgmann, 2000; Rainbow, 2002; Rainbow *et al*., 2004). For predicting toxic effects of metals in sediments for many species, it may be more useful to understand the processes that affect the rate of uptake of contaminants, than to know the net bioaccumulation of contaminants (Rainbow, 2002, 2007; Simpson, 2005; Casado-Martinez *et al*., 2010).

5.3 Choice of biomonitor organism

Bivalve molluscs and oligochaetes are among the most well-established biomonitors of contaminants in marine and freshwater environments (Phillips and Rainbow, 1994; USEPA, 2000a; Robinson *et al*., 2005; OECD, 2007). Although used less intensively for biomonitoring, organisms such as amphipods, chironomids, gastropods, polychaetes, sponges, nematodes and others also have the required attributes to be effective biomonitors (Phillips, 1977; Phillips and Rainbow, 1994; Langston and Spence, 1995; Taylor and Maher, 2003, 2006; Meador, 2006; Waring *et al*., 2006; de Mestre *et al*., 2012; Ding *et al*., 2012; Yu *et al*., 2012).

Phillips (1990) has suggested that for organisms to be effective biomonitors they must be:

- sedentary and therefore representative of the study site;
- hardy and tolerant of high concentrations of contaminants;
- widespread and abundant in the environment being studied;
- easy to identify and collect, with sufficient tissue for analysis of contaminant concentrations; and
- able to accumulate higher tissue concentrations of contaminants in contaminated environments than in uncontaminated environments.

Lee (1998), when discussing methods for the use of marine or estuarine benthic organisms for assessing bioaccumulation in sediments, suggested the following criteria:

- sediment ingester;
- infaunal (preferably non-tubicolous);
- hardy;
- easily collected or cultured;
- sufficient biomass for analysis;
- high bioaccumulation potential;
- feeding behaviour that is understood; and
- suitable for mechanistic/kinetic studies.

There is some commonality between the Phillips (1990) and Lee (1998) criteria. For metal bioaccumulation studies, an organism that ingests sediment is not always required. Organisms that live in sediment and feed near the sediment surface and cause bioturbation will take in metals in the pore waters and bacteria and algae they ingest. Thus these organisms can be effective biomonitors, their bodies having metal concentrations that reflect sediment contamination (Taylor and Maher, 2012a,b,c; Marasinghe Wadige *et al*., 2014a,b). For HOCs that are strongly bound to sediments and probably not found in pore waters, or that are readily transferred to biota, a sediment-ingesting organism is required. If bioaccumulation of contaminants from suspended sediments is to be assessed, for example from a dredging event, a filter-feeding organism that lives in the water column or attaches to a solid substrate, such as an oyster, may be a suitable test species (Edge *et al*., 2014; Schmitz *et al*., 2015).

Organisms to be used for laboratory studies of contaminant bioaccumulation should be able to tolerate a range of conditions, such as of salinity and temperature. Additionally, species should be sufficiently tolerant of contaminants that they can survive relatively long exposure times, and have a low potential for metabolising contaminants (USEPA, 2000b). To be used as a biomonitor, an organism needs to accumulate contaminants, preferably in proportion to the bioavailable concentrations in sediments. This can be established through exposure to laboratory-spiked sediments (Taylor and Maher, 2010) or by measurements of organisms *in situ* along sediment contamination gradients (McCarthy, 1990; Luoma and Rainbow, 2005). The bivalves *Anadara trapezia*, *Tellina deltoidalis* and *Hyridella australis*, for example, have clear relationships of metal uptake relative to the prevailing sediment metal concentrations (King *et al*., 2010; Campana *et al*., 2013; Taylor and Maher, 2013, 2014b; Marasinghe Wadige *et al*., 2014a,b). For HOCs there are numerous studies demonstrating strong relationships between sediment contaminant concentrations (pore-water or sediment concentrations normalised to organic carbon) and bioaccumulation (Meador, 2006; You *et al*., 2011; Ding *et al*., 2012).

When using organisms transplanted in the field or in the laboratory, it is recommended that bioaccumulation studies be conducted for at least 28 days (ASTM, 2010, 2013), as this time is believed to be sufficient for most infaunal benthic species and contaminants to reach steady-state tissue concentrations. This, however, is not always the case. Burt *et al*. (2007) found that 60–90 days were required for *A. trapezia* to reach steady-state tissue metal concentrations. Many species can regulate metals or metabolise organic contaminants, for example PAHs (Maher and Aislabie, 1992), and may give a misleading indication of the bioaccumulation potential of an ecosystem. It is essential, therefore, that bioaccumulation studies include one or more species with very low ability to regulate metals or metabolise organic contaminants.

There is general agreement that metals accumulated over long periods are concentrated in the muscle tissues or stored as granules, and shorter-term accumulations are

concentrated either in the tissues that process metals – for example, gills, hepatopancreas, digestive tissues, liver – or in the gut via ingested food and sediments (Phillips, 1990). For hydrophobic organics, accumulation is mainly in fatty tissues, so accumulated concentrations are usually normalised to lipid content.

Organisms that have most commonly been used internationally as biomonitors of contaminants in sediments (in the field) and to study bioaccumulation (in the laboratory or field) are shown in Table 5.1. However, a far greater range of species could potentially be used to evaluate bioaccumulation, provided the chosen species can be demonstrated to be an effective biomonitor; for example those shown in Table 5.2 for Australia. The ecology of all species chosen for bioaccumulation measurements needs to be fully understood so as to obtain interpretable results. Polychaetes, for example, have a variety of habitats within sediments and can be filter feeders, omnivores or carnivores (Waring and Maher, 2005; Waring *et al*., 2005). Not all of the organisms listed in Table 5.2 have been used to measure bioavailable contaminants in sediments, although organisms such as the oyster *S. glomerata* are

Table 5.1. Common organisms that have been used internationally as biomonitors of contaminants in sediments

Species	Food source[a]	Use[b]	Organs[c]	References
Estuarine–Marine				
Polychaetes		M, O	W	ASTM, 2010; Lee *et al*., 1993, 2001; Millward *et al*., 2005; Morales-Caselles *et al*., 2008; Casado-Martinez *et al*., 2013; Ramos-Gomez *et al*., 2011
Neanthes arenaceodentata	Om			
Capitella capitata	Non-selective DF, coprophagous			
Hediste (Nereis) diversicolor	Om, capable of F			
Arenicola marina	Surface DF			
Nereis virens	Om			
Bivalves		M, O	W	ASTM, 2010; Riba *et al*., 2004; Cheggour *et al*., 2005; Hendozko *et al*., 2010; Tankoua *et al*., 2011; Hylleberg and Gallucci, 1975; Lee *et al*., 1993
Scrobicularia plana	DF			
Macoma balthica	DF, F			
Macoma nasuta	DF			
Freshwater				
Oligochaetes		M, O	W	ASTM, 2010; Higgins *et al*., 2007; Phipps *et al*., 1993; Mendez-Fernandez *et al*., 2013; Mackenbach *et al*., 2012; Ankley *et al*., 1992
Tubifex tubifex	Selective DF			
Lumbriculus variegatus	D			
Amphipods and Midges *Hyalella azteca* *Chironomus tentans*	Selective DF	M, O	W	Borgmann, 2000; Ingersoll *et al*., 1995, 1998; Landrum *et al*., 2004

[a] Om = ominivores, F = filter feeders, D = detritivores, DF = deposit feeders.
[b] M = metals/metalloids, O = organics.
[c] W = whole organism.

Table 5.2. Common organisms that have been used in Australia as biomonitors of contaminants in sediments

Species	Food source[a]	Use[b]	Organs[c]	References
Marine				
Anadara trapezia (bivalve – Sydney cockle)	Om	M	W	Jolley *et al.*, 2004; Burt *et al.*, 2007; Taylor and Maher, 2012a,b,c
Mytilus edulis (bivalve – mussel)	F	M, O	W	Talbot, 1989; Haynes *et al.*, 1995
Tellina deltoidalis (bivalve – deposit feeder)	DF	M	W	Campana *et al.*, 2013; King *et al.*, 2005; Taylor and Maher, 2010, 2013, 2014a,b
Trichomya hirsuta (bivalve – hairy mussel)	F	M	W	Lopez *et al.*, 2014
Saccostrea glomerata (Sydney rock oyster)	F	M, O	W	Hardiman and Pearson, 1995; Scanes, 1996; Scanes and Roach, 1999; Spooner *et al.*, 2003; Robinson *et al.*, 2005; Edge *et al.*, 2014
Mugil cephalus (fish – sea mullet)	D	M	Mu, L, K, G, Go, S, H	Kirby *et al.*, 2001a,b; Waltham *et al.*, 2013
Fish (assorted)	H, D, Om, C	M	Mu, R	Eustace, 1974; Plaskett and Potter, 1979; Marks *et al.*, 1980; Roach *et al.*, 2008
Polychaetes	F, Om, D	M	W	Waring *et al.*, 2006
Suberites sp. and *Mycale* sp. (sponges)	F	M	W	de Mestre *et al.*, 2012
Freshwater				
Hyridella depressa (bivalve – mussel)	F	M	W	Jeffree *et al.*, 1993; Byrne and Vesk, 1996; Adams *et al.*, 1997; Adams and Shorey, 1998
Hyridella australis (bivalve – mussel)	F	O	W	Ryan *et al.*, 1972; Marasinghe Wadige *et al.*, 2014a,b
Velesunio ambiguous (bivalve – mussel)	F	M	W	Jones and Walker, 1979; Millington and Walker, 1983; Jeffree *et al.*, 1993
Velesunio angasi (bivalve – mussel)	F	M	W	Jeffree and Brown, 1992; Ryan *et al.*, 2008; Bollhöfer *et al.*, 2011
Alathyria condola (bivalve – mussel)	F	CY	W	Negri and Jones, 1995
Westralunio carteri (bivalve – mussel)	F	O	W	Storey and Edward, 1989

[a] Om = omnivores, F = filter feeders, D = detritivores, DF = deposit feeders, H = herbivores, C = carnivores.
[b] M = metals/metalloids, O = organics, CY = cytotoxins.
[c] W = whole organism, Mu = muscle, L = liver, K = kidney, G = gills, Go = gonads, S = stomach, H = heart, R = reproductive organs.

known to be useful for measuring bioavailable contaminants in suspended sediments (Edge *et al.*, 2014; Schmitz *et al.*, 2015). As physical suspension, bioturbation and uptake by micro-organisms and algae result in multiple pathways of metal exposure, most organisms listed in

Tables 5.1 and 5.2 will be appropriate for assessing the bioaccumulation of metals. For assessing organics that are strongly bound to sediment particles and not likely to be easily re-mobilised or taken up by microrganisms, and/or for algae, it is best to use organisms that ingest sediments, for example polychaetes or oligochaetes.

5.3.1 Contaminant-specific considerations

Metals

Bioaccumulation of metals in molluscs and other organisms is influenced by physiological factors including age, growth, gender, reproductive condition and genetics. Sampling strategies should aim to minimise the effects of these factors in bioaccumulation studies (for example, comparing organisms of similar age and gender). Sediment properties, redox potential, pH, salinity and temperature are factors that affect metal bioavailability (Brown and Depledge, 1998). These factors also influence the physiological activity of organisms through their effects on metabolic rates (Frazier, 1976). It is necessary to understand the effects of all of these factors on metal accumulation in organisms because they may lead to variations in tissue metal concentrations that if not accounted for will make results difficult to interpret.

It has been established that the tissue metal concentrations may vary with mass and size of molluscs (Taylor and Maher, 2003). Temporal fluctuations in mass and metal body burdens may also occur (Robinson *et al*., 2005; Taylor and Maher, 2006). For some organisms, tissue metal and metalloid concentrations can remain relatively constant – selenium for example – suggesting that the organisms have reached equilibrium with their environment (Taylor and Maher, 2012c).

Many studies have found a trend of decreasing tissue metal concentrations with increased tissue mass (Boyden, 1977; Lobel *et al*., 1991; Langston and Spence, 1995) although this does not always occur (Cubadda *et al*., 2001). This 'dilution effect', where growth dilutes the metal content, appears to be a common phenomenon in molluscs. It is also postulated that where mass is independent of tissue metal concentration, there may be some form of regulation of uptake and excretion (Phillips and Rainbow, 1994). An examination of data comparing mass and metal concentration shows that a minimum of three orders of magnitude in mass is required before a significant relationship between mass and tissue metal concentration is evident in bivalves.

Age may also influence metal concentrations: older organisms show less variability, probably because of high metabolic activity in juveniles. Robinson *et al*. (2005) found there was less variability between individual oysters' metal concentrations once they entered adulthood, and suggested that this is due to biochemical changes occurring during rapid faster growth in juveniles. For testing, collection of mature individuals would reduce the within-sample variability, but size is not necessarily a good indicator of age and therefore often it is not possible to sample mature individuals.

Genetic differences may cause an overlap in the distributions of tissue metal concentrations in contaminated and uncontaminated locations. In other words, individuals from a contaminated environment may accumulate lower metal concentrations than individuals from uncontaminated environments (Taylor and Maher, 2003). It appears, for example, that some molluscs may take up metals at a lower rate, or they may have enhanced regulatory mechanisms (Lobel *et al*., 1982, 1991).

Gastropods from contaminated sites had a higher degree of individual variability in metal concentrations than those from an uncontaminated site in studies by Taylor and Maher (2003). Positive skewness in the distributions of metal concentrations occurs in populations of gastropod and bivalve molluscs from both contaminated and uncontaminated environments

(Taylor and Maher, 2003; Robinson *et al*., 2005). Although some individuals from contaminated environments accumulate lower concentrations of metals than individuals in an uncontaminated environment, the majority do not. At the individual level there can be a large range in tissue metal concentrations, while at the population level the distributions are separate (Taylor and Maher, 2003). Some individuals may either be taking up less metal or have enhanced excretory mechanisms, and others are accumulating excessive quantities of metals, but the majority of the population falls somewhere in the middle. These results (Taylor and Maher, 2003; Robinson *et al*., 2005) could not be explained on the basis of either mass or gender or habitat differences. Skewness seems to be a common factor in sample distributions of natural populations because of the natural variation between individuals. Collecting greater numbers of controls/reference organisms may help inform the test laboratory about skewness and improve the power of the study in determining if differences between populations are significant. In summary, molluscs will reflect the levels of biologically available metals of their respective environments, and, in the contaminated environment, they are net accumulators. Variances usually increase as mean metal concentrations increase.

Significant differences found in metal concentrations due to gender, where they exist, have been thought to be associated with spawning (Lobel *et al*., 1991) because metal concentrations vary as mass fluctuates when oocytes are produced and shed.

Collecting organisms that are at different stages of spawning may also contribute to differences between individuals and hence overall variability (Simpson, 1979; Cossa *et al*., 1979). The use of triploid oysters, which do not reproduce, has been shown to considerably reduce variability in observed metal concentrations (Robinson *et al*., 2005).

Organics

For non-ionic HOCs, the lipid content of an organism is an important factor in its contaminant uptake and storage. Lipid content can vary considerably within a single species, based on life stage, gender, sexual maturity and season, and this will affect the bioaccumulation of organic contaminants (Moore *et al*., 2005).

Organic contaminant concentrations are likely to vary with mass and size of molluscs (USEPA, 2000). Greater mass and size are often a reflection of age and it would be expected that older organisms would have had longer exposure times and exhibit greater contaminant concentrations. Gender can also be expected to have significant effects on organic contaminant concentrations. For example, during spawning there are fluctuations in the mass of an organism, especially its gonads (Meador *et al*., 1995). Collection of organisms that are at different stages of spawning would contribute to differences observed between individuals and hence overall variability (Bruner *et al*., 1994, Meador *et al*., 1995).

Genetic differences may also accentuate the individual variability in organic contaminant concentrations which occurs in natural populations and skews the natural distributions of contaminant concentrations.

Organic contaminant content can fluctuate through time, related to an organism's feeding behaviour and reproductive cycle. Many organisms feed more extensively and grow more rapidly during warmer periods (for example in summer) and thus lipid content may be greater at these times than at other times when lipid reserves are used. As well, lipid content can increase as gonadal tissues grow. On spawning, this gonadal material will be lost (Bruner *et al*., 1994).

Selection of sample size

When comparing locations, it is important to select a large enough sample size to gain a true estimation of the mean bioaccumulated contaminant concentrations and to allow

concentration differences to be detected. Inherent variability existing in organism populations may obscure trends and lead to incorrect conclusions. Sample size calculations are relatively easy to perform (Zar, 1984), but the concentration differences required to establish social or environmental significance are a management decision. Three simple procedures can be used to calculate sample size:

- sub-sampling the dataset using a random number table to determine the number of replicates required to obtain a value within 10% of the population mean (Taylor and Maher, 2003);
- using the means and standard deviations of contaminant concentrations and Student's *t* tables as outlined by Zar (1984) (Taylor and Maher, 2003);
- using bootstrap analysis of metal concentration data to obtain the 95% confidence interval for discrete sample sizes (Robinson *et al*., 2005).

For oysters, for example *Saccostrea glomerata*, and gastropods, for example *Austrocochlea constricta* and *Bembicium auratum*, although the inherent variability is large, only 10 samples need be analysed to obtain an estimate of the mean concentration within ±10% of the population mean (Taylor and Maher, 2003; Robinson *et al*., 2005). This sample number should allow changes of 30% from the mean contaminant concentration to be detected.

Many studies have used pooled samples (that is, they combine multiple individual samples to provide a composite) to reduce variability and reduce analysis costs. The disadvantage is that information on contaminant variability is lost. If subtle changes in bioaccumulation are occurring they are often seen as increases in contaminant variability rather than increases in means. As well, skewing of the means of contaminant concentration can occur if a few samples contain high concentrations of contaminants, especially metals. This cannot be detected if pooled samples are used.

As a consequence of the above, when collecting organisms, attention should be given to the following factors to minimise variability and aid in interpretation of results.

- Mass. Organisms of similar mass and size should be selected from all locations. A regression analysis should be used to establish that tissue contaminant concentrations are independent of mass (or size). If mass or size dependence is established, then concentrations should be normalised to a chosen mass.
- Gender. Most studies have found that gender *per se* is not a large contributor to metal concentration variability; but known periods of spawning should be avoided because contaminants may be lost during this time. For HOCs, gender differences may be associated with lipid content differences and collection should be standardised to female or male organisms.
- Genetics. Inherent variability appears to be a 'universal characteristic' of contaminant concentration distributions particularly in molluscs; thus sufficient replicate samples should be collected to account for this variability. All measured contaminant concentrations should be used in statistical analyses because high contaminant concentrations at uncontaminated locations are not outliers.
- Accumulation of chemicals of interest at contaminated and uncontaminated locations. Preferably, organisms should accumulate contaminants in direct proportion to the contamination in sediments. If organisms are to be used as biomonitors, it must be established that these organisms accumulate higher tissue concentrations of contaminants in contaminated environments than in uncontaminated environments.
- Temporal variation. For comparison purposes, organisms from contaminated and uncontaminated locations need to be collected after similar exposure periods and at similar times.

- Sample size. Sample size needs to be large enough to produce a true estimation of the mean contaminant concentrations and to allow concentration differences to be detected. Sample size calculations are relatively easy to perform (Zar, 1984), but concentration differences required to reveal social or environmental significance are a management decision. Where there is insufficient mass of organisms to provide a large enough sample, pooling of samples is an option, noting the limitations discussed earlier.

5.3.2 Choice of tissues or sub-cellular fractions to be measured

Metals

Bioaccumulated contaminants will be distributed within the test organism, depending on the major route of uptake, the site and mechanism of storage, and the mechanism of excretion. The distribution is very significant for assessments because it is now recognised that, for example, metals bound at different sites have different functions, and impairment of function is the potential cause of toxicity within an organism. Thus, organisms in the field that have been accumulating metals for a long period may be storing most of the metal in a detoxified form. Toxic effects are elicited when a critical dose of a chemical is reached in one or more sensitive compartments of the organism, or toxicity may take effect if the metabolically available concentration exceeds a threshold concentration, or if the metal influx rate exceeds the combined rates of detoxification and excretion (Rainbow and Luoma, 2011).

Most dietary-derived metals are processed in the gut and then accumulated internally and stored in the hepatopancreas or other tissues as granules; they may be excreted via the liver. Metals taken up directly from the water column are concentrated in the gills, hepatopancreas and mantle. Excretion may occur by a range of processes: via direct egestion, excretion through the gills, or via the liver (Luoma and Rainbow, 2008). While for most applications the data from the bioaccumulation line of evidence will be based on whole body concentrations, more detailed examination of tissue distribution can add value.

In bivalves, the distribution of a metal at the sub-cellular level can be measured in the gill and hepatopancreas to determine metabolically active and detoxified metal fractions, and used to interpret biological effects (Taylor and Maher, 2010). Biological effects are related to the threshold concentrations of metabolically-available metals and not to total accumulated metal concentration (Rainbow, 2002; Vijver *et al.*, 2004; Simpson and King, 2005). A scheme for separating operationally defined sub-cellular fractions in molluscs is given in Fig. 5.1. Typically, information can be gathered on five major sub-cellular fractions of metals within organisms:

- metal-rich granules (MRG);
- nuclei and cellular debris;
- organelles (ORG), including mitochondria, microsomes and lysosomes;
- heat-denaturable proteins (HDP), also referred to as heat-sensitive proteins; and
- metallothionein-like proteins (MTLP), also referred to as heat-stable proteins.

The biologically active metal fraction (BAM), which combines ORG and HDP, is the target of attack of metals in cells, and a biologically detoxified metal fraction (BDM), which combines MRG and MTLP, is considered to alleviate toxicity.

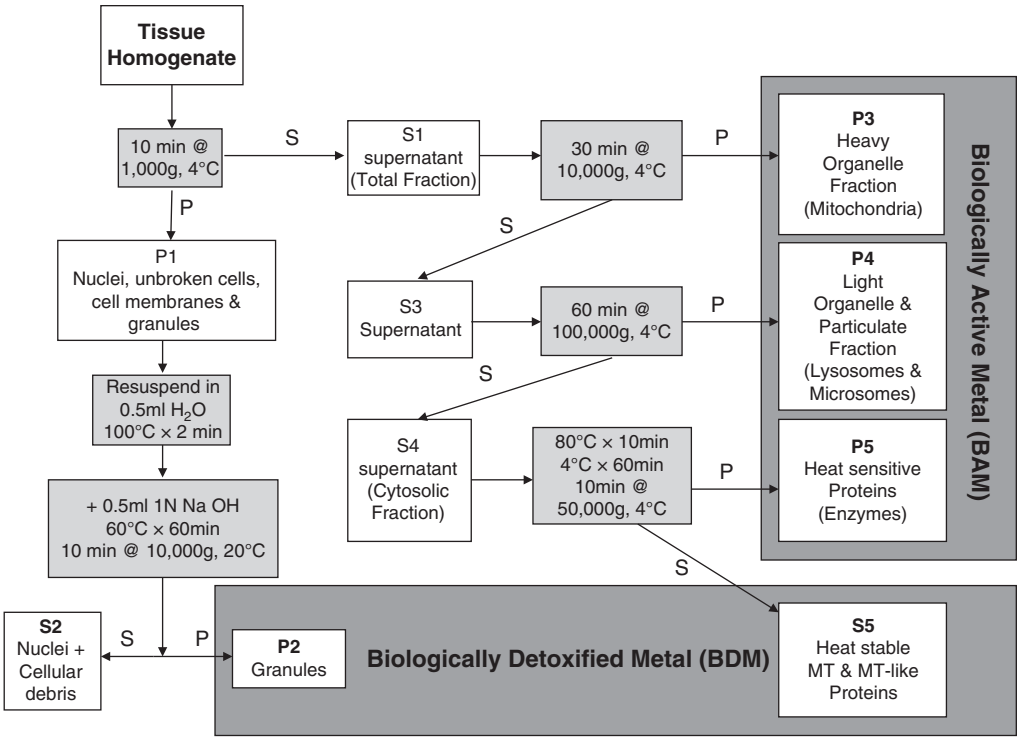

Figure 5.1. Procedure for sub-cellular fractionation of bivalve tissues by differential centrifugation. The shaded boxes show details of the centrifugation and digestion or heating steps used to obtain the specific fractions. The final fractions – four pellets P2, P3, P4 and P5 and two supernatants S2 and S5 – are grouped as: biologically detoxified metals (BDM) P2 and S5; and biologically active metals (BAM) P3, P4 and P5; or as S2 which contains metal associated with dissolved tissues (Taylor and Maher, 2012b). MT = metallothionein.

Thus consideration should be given to:

- selection of tissues; for general monitoring purposes analysis of whole tissues is sufficient. If a deeper understanding of the pathway of contaminant uptake is required, individual tissues need to be analysed to ascertain the route of uptake. Metals such as cadmium and zinc found accumulated in gill tissues, for example, are generally attributed to water, while metals/metalloids such as Se, Hg and Pb found accumulated in digestive tissues are from food;
- need for sub-cellular fractionation; if the focus is on relating bioaccumulated contaminants to effects, then measuring contaminants (for example metals in sub-cellular fractions) provides additional information for understanding the mechanism of toxicity. This information can also be used to explore the transfer of contaminants from prey to predator organisms if food web interactions or ecosystem questions relating to contaminants are being explored.

Organics

For bioaccumulated organic contaminants, a major limitation is analytical detection. A requirement is that the tissue mass be sufficient for chemical analyses (Exponent, 1998).

The analysis of the concentrations in specific tissues will therefore be restricted to larger organisms such as fish. Only where the concern is for human health effects would tissues be sub-sampled to reflect the concentrations of contaminants in the parts that are consumed. Additionally, specific organs might be sampled where the mechanism of bioaccumulation is a concern (Bruner *et al*., 1994; Meador *et al*., 1995). For a weight-of-evidence assessment, because HOCs associate with lipid tissue, it is usual to use only the whole organism for analysis. Bioaccumulated concentrations are usually expressed on a wet weight basis or normalised to lipid content.

Methods used for lipid analysis have been summarised by Schlechtriem *et al*. (2012).

5.4 Choice of approach

5.4.1 Field collection versus transplantation studies

There are three approaches that can be used to assess contaminant bioaccumulation in field or laboratory experiments:

(i) passive biomonitoring: the measurement of contaminant concentrations in indigenous organisms;
(ii) field transplantion (active biomonitoring): where organisms are transplanted into contaminated environments and their contaminant concentrations are measured after a specified time;
(iii) laboratory transplantion (active biomonitoring): where organisms are transplanted into microcosms containing contaminated sediments in the laboratory and their accumulated concentrations are measured after a specified time.

Each approach has advantages and disadvantages. Passive biomonitoring requires organisms to be present at the sites of interest in sufficient numbers for statistical analyses. Contaminant concentrations will reflect exposure over the lifetime of the organism and will account for population adaptation to contaminated environments, so may exhibit fewer effects. A disadvantage is that organisms will be genetically different, introducing some inherent variability into contaminant concentrations.

Field transplantation allows genetically similar organisms to be put into depauperate environments and for the uptake of contaminants to be measured in absolute terms, and rates of uptake calculated. Organisms are also subject to real environmental variability (temperature, pH, dissolved oxygen, redox potential and food) that may influence the uptake of contaminants. A limitation is that organisms will not have adapted to contaminated environments and uptake, and effects may be greater than for indigenous organisms. Another limitation is the lack of security when deploying caged organisms in populous areas.

Laboratory transplantation also allows the uptake of contaminants to be measured in genetically similar organisms, in absolute terms, and rates of uptake to be calculated. The major limitations are that organisms will not be subject to real environmental variability and will experience potentially artificially elevated contaminant exposure via the overlying water, and so the study may over- or under-estimate uptake. As well, sediment properties influencing contaminant variability (for example pH, dissolved oxygen, redox potential) may be affected by removing sediments from their natural environment, so sufficient time for their re-equilibration (7–10 days) is required. The main advantages

are the security that the laboratory setting gives, and the opportunity to control extraneous variables.

5.4.2 Study design and statistical analysis

The three approaches are illustrated in a study using the bivalve *Anadara trapezia* to measure the bioaccumulation of metals from contaminated sediments. This study focused on the estuarine Lake Macquarie, New South Wales (NSW), Australia. Similar protocols would be employed for the determination of HOCs in bivalves or other biota.

Lake Macquarie, NSW, Australia – an example

Industrial development around Lake Macquarie (Fig. 5.2) is extensive and consists of a decommissioned lead–zinc smelter, a fertiliser plant, a steel foundry, collieries, sewage treatment works and two coal-fired power stations. The lake supports a large recreational fishery and is also of ecological significance, providing breeding and nursery grounds for many commercial fish species. In comparison to other NSW estuaries, Lake Macquarie has significantly higher concentrations of metals, particularly lead and the metalloid selenium, in its sediment (Roy and Crawford, 1984; Peters *et al*., 1999b; Kirby *et al*., 2001b). The lead–zinc smelter to the north of the lake was in operation between 1897and 2003, and is a known source of Zn, Se, Cd and Pb (AWACS, 1995). There is a clear contamination gradient for Cu, Zn, Cd and Pb in the northern part of the lake (Burt *et al*., 2007). Metal concentrations above background levels in the southern reaches and selenium hot spots near power stations indicate that the coal-fired power stations are also contributing selenium and metals to the lake (Peters *et al*., 1999a).

The locations in Lake Macquarie from which indigenous organisms were collected and which were used for field and laboratory sediment transplant experiments are indicated in Fig. 5.2. Lead concentrations in sediments at these locations are shown in Fig. 5.3, together with values for three reference locations. The Sydney cockle, *Anadara trapezia*, was chosen for assessing bioaccumulation and effects because it is a sediment-dwelling organism native to Lake Macquarie and has been shown to accumulate metals in areas where sediments are contaminated with metals (Furner, 1979; Batley, 1987; Burt *et al*., 2007).

Field sampling (passive biomonitoring)

Experimental design principles

A typical field study will collect organisms from several locations to assess and rank bioaccumulation. If only one location is of interest, this location will need to be assessed relative to at least three reference locations. Replication within locations ('sites') is required to enable statistical analysis. Thus studies will need to be designed to enable a two-factor nested analysis of variance ('factors': location and site). Locations are the general areas of sampling; sites are specific areas at least 100 m apart, randomly chosen within locations for sampling. Typically 10–20 organisms are collected by hand for analysis at each site.

Study sites, results and analysis

Maher *et al*. (1998, unpublished data) collected *A. trapezia* from four locations in the northern part of the lake (Fig. 5.2) and three reference locations in nearby estuaries (Bagnalls Beach, Tea Gardens and Smiths Lake). Lead concentrations in whole tissues were significantly different among locations within the lake, with mean concentrations

Figure 5.2. Map of Lake Macquarie NSW. The seven study locations were Cockle Creek 1, Cockle Creek 2, Cockle Bay, Warners Bay, Kooroora Bay, Eraring Bay and Whiteheads Lagoon. The lake is connected to the Pacific Ocean at Swansea.

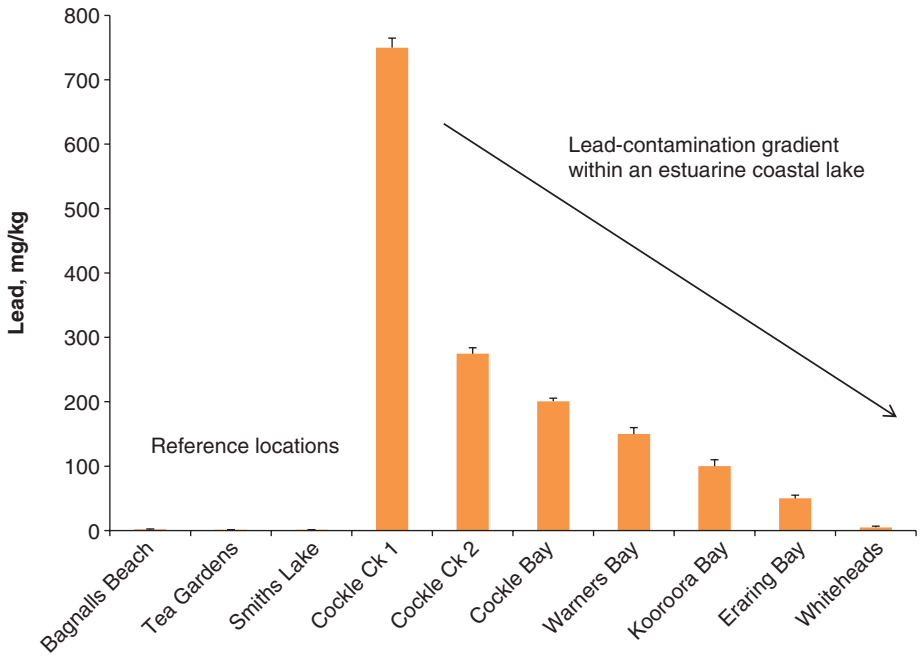

Figure 5.3. Concentrations of lead in sediments of Lake Macquarie and reference locations.

decreasing in the order Cockle Bay > Warners Bay > Kooroora Bay > Eraring Bay, and greater than at the three reference locations (Fig. 5.4). The bioconcentration factors were 33, 12, 9 and 3 for cockles collected from the respective sampling locations.

Field transplantation

Experimental design principles

Studies with caged organisms in the field need to be designed to allow a four-factor nested analysis of variance (factors: time, location, site and cage). Locations are the general areas of sampling; sites are specific areas within locations, at least 100 m apart and randomly chosen for cage deployment. A typical design might consist of three sampling times (30, 60 and 90 days), three or four locations (including control), two sites nested in each location and two cages nested in each site. The design can be simplified by choosing one time – typically 60 days for *A. trapezia*, and 28 days for other bivalves. If a time component is included, at least two randomly chosen cages need to be retrieved at each time from each site, nested within the locations. Organisms to be caged are collected from a reference location and transported in a portable cooler box containing sediment and water from the collection site and an aquarium air pump to aerate overlying water during transportation and maintain ambient temperature. Typically 10–20 organisms are placed in each cage.

Choice and deployment of cages

Previous studies (Cain and Luoma, 1990; Martinčić *et al*., 1992; Couillard *et al*., 1995; Dewitt *et al*., 1999) have concluded that cages *per se* have no treatment effects. The cages

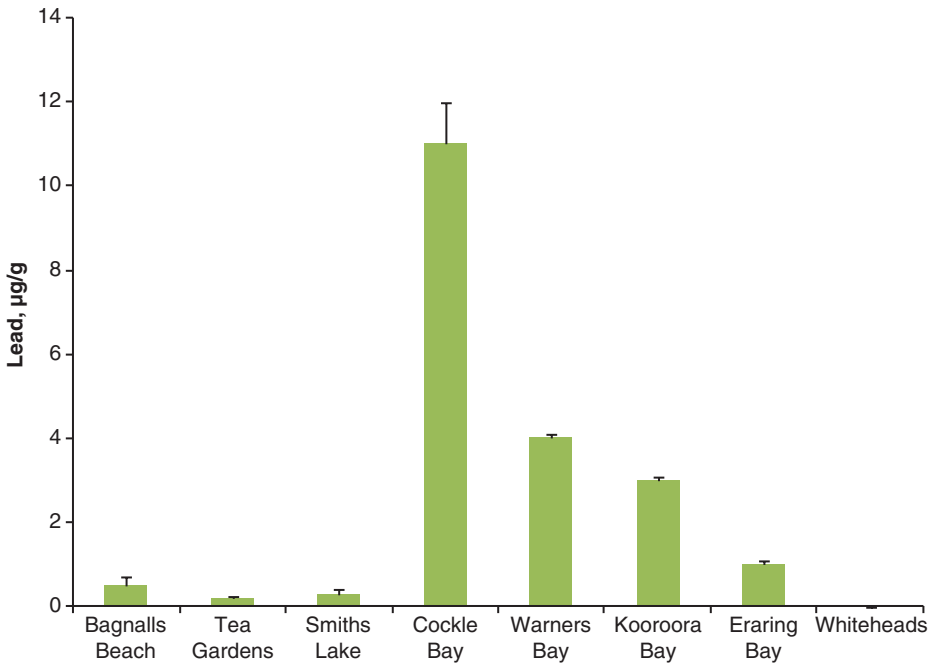

Figure 5.4. Concentrations of lead in tissues of indigenous *Anadara trapezia* from Lake Macquarie and reference locations.

may need to be deployed by Scuba divers, depending on the water depth at the sites of interest. In design, the cages should:

(i) be large enough to hold the number of organisms required, with adequate access to sediments;
(ii) allow flow-through of water;
(iii) be of non-contaminating materials; typically, plastic oyster cages have been used (see Fig. 5.5); and
(iv) be secured to avoid predation and escape of organisms; oyster trays with netting have been used for this purpose (Fig. 5.5).

A consideration, based on the question to be answered, is whether cages are to be buried in sediments or placed on the sediment surface. If cages are buried in sediments, organisms will be exposed to metals through ingestion (sediment particles and food), dermal absorption and metals released by bioturbation. If cages are placed on sediments, organisms will only be exposed to metal through ingestion of food and metal fluxes.

The organisms chosen will also influence how cages are deployed. Most oysters and mussels are filter feeders and do not have to be in sediments. Other organisms, such as *A. trapezia* and *Ostrea angasi* (mud oyster), live in sediments and need to be able to bury themselves to function. *Tellina deltoidalis* is a deposit feeder and feeds from the sediment, so also requires sufficient sediment to burrow and feed.

A period of 60–90 days is sufficient for *A. trapezia* to reach equilibrium with their environment (Burt *et al.*, 2007), but this equilibration period needs to be determined for each species of organism chosen. Individual cages and organisms needed to be marked (for example using adhesive numbers) to allow an assessment of the condition of the organisms (mass:volume ratio) at the end of the deployment period.

Figure 5.5. Field transplantation cage, about 60 cm × 40 cm × 10 cm.

To avoid vandalism, cages need to be fully submerged to at least a depth of 1 m with no visible markers. To retrieve the cages, the locations need to be accurately known. A simple method of achieving this in shallow waters near the shore is to attach two ropes to known points on the shore and use these to triangulate the cage positions. In deeper waters, a GPS device may be needed to locate cages.

Study locations, results and analysis

Burt *et al.* (2007) and Taylor and Maher (2011 unpublished) transplanted *A. trapezia* to seven locations in Lake Macquarie (Fig. 5.6). Lead concentrations in whole tissues reached a maximum after 2 months and were significantly different among locations. Mean lead concentrations decreased in the order Cockle Creek 1 > Cockle Creek 2 = Cockle Bay > Warners Bay > Kooroora Bay > Eraring Bay > Whiteheads Lagoon and were significantly higher than the reference locations Bagnalls Beach, Tea Gardens and Smiths Lake (Fig. 5.6). The bioconcentration factors were 67, 41, 42, 18, 9, 6 and 3 at the respective locations, and were similar to those of indigenous *A. trapezia*.

Laboratory transplantation – microcosm exposure

Experimental design principles

A typical laboratory study will compare the bioaccumulation of metals by organisms in laboratory microcosms using sediments collected from a gradient of contamination (minimum three sites). Again, if only one location is of interest, this location will need to be assessed relative to at least three reference locations. Replication within locations is required to enable statistical analysis. Studies need to be designed to enable either a one-way (factors: location and replicates) or a two-way analysis of variance (factors: location, time and replicates).

Sediment, seawater and organism collection

Sediments are collected with a stainless-steel spade and press-sieved through a 2 mm stainless-steel mesh to remove rocks, large pieces of organic material and organisms. The sediments are

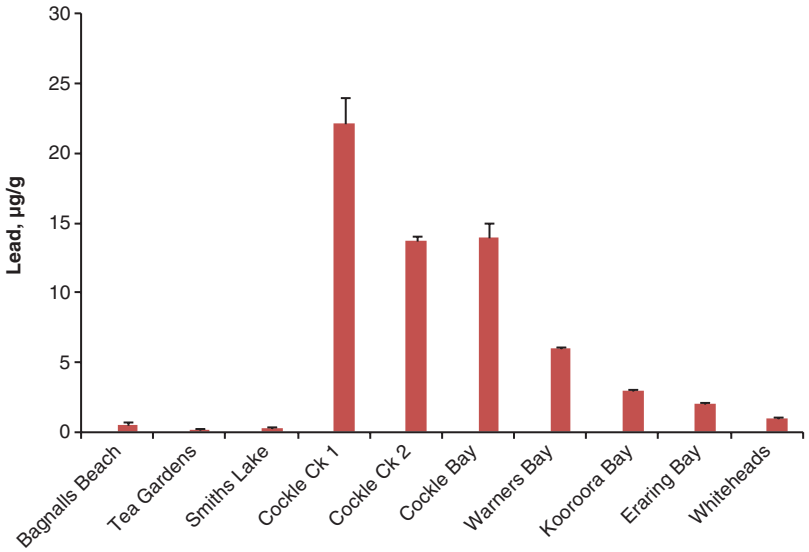

Figure 5.6. Concentrations of lead in tissues of *Anadara trapezia* field-transplanted in Lake Macquarie and from reference locations.

placed in a series of plastic buckets with lids and sealed with tape for transport. Sediments can be stored at 4°C for up to 2 months. Before use in experiments, the sediments are sub-sampled for analysis of metal concentrations, to determine metal exposures. Moisture content, salinity, pH and sediment grain size are also measured to establish the physico-chemical properties of both the control and the test sediments. Properties of control and test sediments should be closely matched to ensure the results for contaminant dose and effects are not influenced by physico-chemical factors, rather than the contaminants of interest.

Seawater is collected from uncontaminated coastal waters and adjusted with deionised water to match the salinity of locations where test organisms are being collected: usually 30 ± 2‰.

Test organisms are collected from reference locations and placed in portable cooler boxes containing sediment and water from those sites, with aquarium air pumps to aerate overlying water during transportation and maintain ambient temperature. The organisms are maintained in aquaria, with control sediments 10 cm deep and water of the same salinity as the collection site, for up to 2 weeks to acclimatise before experimentation. Overlying waters are aerated using in-line control valves on air hoses to achieve ≥85% oxygen saturation without disturbing sediments. Water temperature is maintained at 22 ± 1°C and the photoperiod is 14-h light : 10-h dark. If ambient water temperatures at the time of collection are 10 ± 5°C cooler or warmer than 22 ± 1°C, the water temperature of the holding tanks is adjusted gradually by 2 ± 0.5°C per day until the experimental temperature is reached. A 3-day feeding/half water-change cycle is maintained during the acclimatisation period using a suitable supplementary food such as the unicellular green algae *Nannochloropsis* preparation (Nanno 3600, Instant Algae®, USA).

Laboratory microcosms

Typical microcosms are 10–12 L glass or polystyrene containers or aquaria containing 1000 g of wet sediment (with a minimum of 20% <63 μm fraction) and 8–10 L of seawater (Fig. 5.7). Overlying waters are aerated using in-line control valves on air hoses to achieve ≥85% oxygen saturation without disturbing sediments. As in the holding tanks, water temperature is maintained at 22 ± 1°C and the photoperiod is 14-h light : 10-h dark (3.5 μmol photons/s/m^2).

Study, results and analyses

Taylor (2009) used microcosms to expose *A. trapezia* to sediments collected from three locations in Lake Macquarie, representing a sediment lead gradient (Cockle Creek sites 1 and 2 and Cockle Bay; Fig. 5.3). Three replicate microcosms of each treatment and of the control sediment were set up, each containing 15 *A. trapezia*. Organisms were exposed to the sediments for a total of 60 days.

Anadara trapezia accumulated lead from the three exposure treatments compared to the control organisms (Fig. 5.8), indicating that lead in the sediment was bioavailable and could be accumulated by this organism. After 60 days, tissue lead concentrations were significantly different between locations. Regression analysis showed significant positive correlations between lead concentrations in *A. trapezia* whole tissue and sediments from Lake Macquarie. The bioconcentration factors were 139, 50 and 35, which was higher than in transplanted and indigenous *A. trapezia* from the same locations in Lake Macquarie.

Comparison of approaches

Comparison of the assessment procedures shows that the collection of indigenous organisms and use of transplantation gave similar results for the bioaccumulation of lead in *A. trapezia*. The laboratory accumulation experiment gave results similar to those from the

other approaches, except for the sediment with the highest lead concentration. Although the laboratory results did not exactly replicate the field results, they did provide a consistent ranking of locations in terms of potential bioaccumulation of lead.

5.5 Sample collection, preparation and analysis

Sediment-dwelling organisms

Species that live buried in sediment, such as polychaetes and some bivalve molluscs, are obtained by sieving. The top 10–20 cm layer of sediment is collected, using a stainless-steel

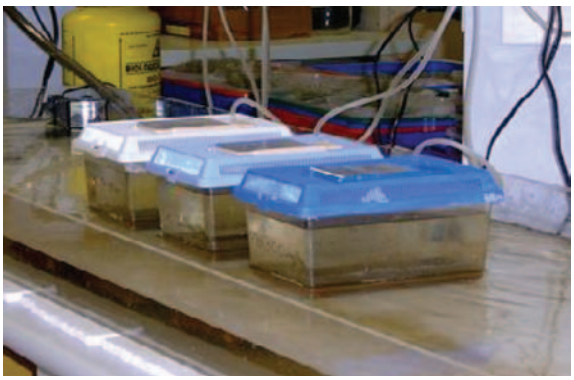

Figure 5.7. Laboratory microcosm exposure set-up.

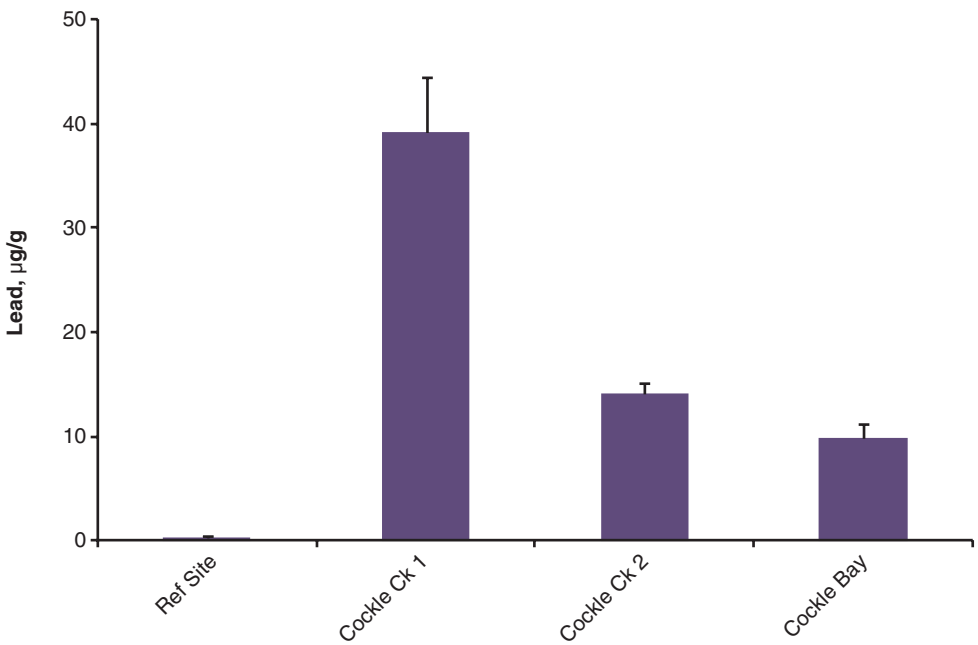

Figure 5.8. Concentrations of lead in tissues of *Anadara trapezia* in the laboratory microcosm study.

shovel, and either a 2 mm- or 1 cm-mesh stainless-steel sieve is used to separate the organisms (depending on organism size).

Organisms that live at the sediment surface can be collected by hand, wearing plastic gloves and locating the bivalves by gently sweeping the fingers through the surface sediments. Extraneous material adhering to mollusc shells is removed by gently scrubbing the shell with a nylon brush and rinsing it in collection water.

Live specimens are transported in a portable cooler box containing sediment and water from the collection site. An aquarium air pump is used to aerate overlying water during transportation and to maintain ambient temperature. For organisms used in laboratory-uptake studies, the field-collected organisms are held in aquaria for up to 2 weeks to acclimatise before experimentation.

Before being analysed for contaminants accumulated at their indigenous location in the field, all organisms normally should be depurated for at least 24 h in clean aerated water from the location where they were collected. If organisms are not depurated, contaminants in gut contents that have not been taken into the organisms' tissues (that is, have not bioaccumulated) will be included in analyses. Organisms are only not depurated when an estimate of total contaminants consumed by predators (and humans) is required.

After depuration, organisms such as polychaetes are frozen and stored at –20°C until analysis. For molluscs, soft tissue is removed from shells and either whole organisms or individual tissues are frozen and stored at –20°C until analysis.

Fish

For sediment assessments, only fish species that are bottom feeders are used. These species are directly affected by sediment particles, pore waters and ingested benthic biota. Fish are collected using nets or electrofishing, and need to be killed by passing a needle into the brain ('pithing'). They are stored individually in sealed plastic bags before freezing on-site using dry ice.

Fish are dissected using stainless-steel dissecting implements. Selected tissues are removed, placed in plastic vials and frozen at –20°C until analysed.

Individual versus composite samples

As mentioned previously, many studies use pooled tissue samples from several individual samples (whole organisms or individual tissues) to reduce variability and reduce analysis costs. The disadvantage is that information on contaminant variability is lost. If subtle changes in bioaccumulation are occurring, these are often seen as increases in contaminant variability rather than increases in means.

Metals

For analysis of metals, the tissue and sediment samples are normally freeze-dried and ground. Sub-samples are typically digested with concentrated acids (for example, nitric acid or *aqua regia*) using microwave heating (Baldwin *et al*., 1994) and the digests are analysed by inductively coupled plasma mass spectrometry (Maher *et al*., 2001). The recovery of metals from relevant certified reference materials (CRMs) can guide the choice of acid mixture.

Organics

Unlike metals, for organics there are particular procedures for extraction, concentration and analysis specific to the organic contaminant of interest, and measurement of lipid content is also often useful. It is outside the scope of this document to provide details of

these methods; guidance should be sought from qualified analysts with experience in analysing the organic contaminant of interest.

Quality assurance/quality control

Digestion, extraction and measurement processes are all subject to errors, including contamination, degradation, matrix effects and calibration errors. Certified reference materials are available to assist in quantifying full procedural errors (see Chapter 3, Section 3.4). In the absence of CRMs, inter-laboratory studies also provide a means of assessing the quality of results. For a full discussion of quality assurance/quality control procedures consult the *Australian guidelines for water quality monitoring and reporting* (ANZECC/ARMCANZ, 2000).

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6

Biomarkers

Anne M. Taylor and William A. Maher

6.1 Introduction

Biomarkers are measurable molecular, cellular and physiological responses within an organism, in relation to single or multiple environmental stressors. The responses can be both chemical and non-chemical (Fig. 6.1). In sediment quality assessments, biomarker effects on benthic organisms can serve as an additional line of evidence as part of a weight-of-evidence assessment (Martín-Díaz *et al.*, 2004; Taylor and Maher, 2010; Schettino *et al.*, 2012; Hook *et al.*, 2014). Biomarker responses, particularly at the molecular and sub-cellular level can be rapid, so they are able to provide early warning of adverse effects (Lam, 2009). Biomarker responses are typically observed in the sub-lethal toxicity range, as precursors to effects on growth, reproduction and survival (Smit *et al.*, 2009). They include subtle changes in normal physiological function such as reproductive behaviour, resilience to disease, and prey-capture abilities, which can have profound impacts on an organism's longer-term survival and reproductive output; ultimately, these can affect ecosystem health (Connon *et al.*, 2009).

It can be difficult to demonstrate the significance of biomarker responses in the health of an organism, and to extrapolate that to longer-term effects on ecosystem health, although this chapter includes examples where this has been done successfully. In assessment programs, biomarkers are significant because they are early warning indicators that frequently occur before higher order effects are observed. To integrate biomarkers into environmental monitoring programs, it is necessary to collect data to improve understanding of both exposure and dose, and to provide the scientific basis on which to interpret biomarker responses in selected species. For a biomarker monitoring program to be useful in a given situation, the chosen biomarkers must be demonstrably capable (in principle) of indicating exposure to the contaminant. Further, the biomarker responses must be able to be linked to effects on individuals and the ecosystem (Fig. 6.1).

Although in environmental toxicology the impacts on populations or communities are of interest, effects at that level typically occur as a result of changes in the fitness of many individuals. For an individual organism, exposure to contaminants can be harmful because they change the individual's normal physiology, and these changes can often be measured at the molecular or sub-cellular level. Damage at the molecular or sub-cellular level results from changes in enzymatic processes, protein expression and function, mutagenesis or cellular membrane degradation leading to cell death (see Table 6.1). These changes occur over a short time frame (hours to days). When considered an 'early warning system'

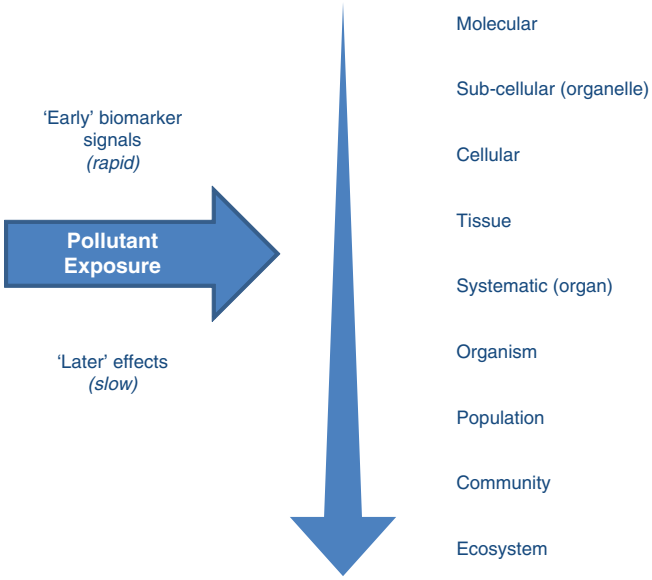

Figure 6.1. Sequence of biomarker responses to exposure to a contaminant (modified from van der Oost *et al.*, 2003).

of damage likely to result in population or community effects (Galloway *et al.*, 2004a,b), they allow action to be taken at an early stage to prevent irreversible ecosystem problems.

Thus biomarkers can help bridge the gap between exposure and effects, providing necessary information to evaluate subtle long-term effects. Biomarkers that comprise biochemical, physiological and histological endpoints may be categorised as either 'biomarkers of exposure', which reflect an organism's attempt to compensate for, or tolerate, stressors in their environment, or 'biomarkers of effect', which reflect deleterious sub-lethal organism effects which may be causally linked to one of the four levels of biological organisation: biochemical and cellular, whole organism, population, and community (Chapman, 1995) (Fig. 6.1).

6.2 Biomarkers of exposure

Biomarkers of exposure include those that are both specific and non-specific for a compound or chemical class, and indicate that exposure to some exogenous chemical has occurred. They may represent either general or specific responses, and have the advantage of quantifying only biologically available toxicants (Mayer *et al.*, 1992).

Biomarkers of exposure reflect an interaction between the xenobiotic agent and a target molecule or cell that is measured in a compartment within an organism (van der Oost *et al.*, 2003) (Table 6.1). Physiological, reproduction and genotoxic biomarkers generally respond to a range of contaminants including metals and organics, while others respond to specific chemical classes; for example metallothioneins to metals, cytochrome P450 to organics, acetylcholinesterase to pesticides, and vitellogenin to estrogens (Table 6.1).

6.3 Biomarkers of effect

Biomarkers of effect can be used to demonstrate an effect resulting from contaminant exposure or other stressors, and include measurable biochemical, physiological or other

alterations within an organism's tissues or body fluids, which can be associated with health impairment or disease (van der Oost *et al*., 2003). 'Biomarkers of susceptibility' have also been defined as a separate category (WHO, 1993). These help to elucidate variations in the degree of response to contaminant exposure observed between different individuals, and include genetic factors and changes in the receptors that alter the susceptibility of an organism to a specific contaminant exposure. Glutathione depletion, for example, has been shown to increase the sensitivity of the oyster *Crassostrea virginica* to copper and reduce its reproductive success (Conners and Ringwood, 2000; Ringwood and Conners, 2000). Effects biomarkers may be direct measures of enzymatic, cellular or physiological change (for example, DNA damage, acetylcholinesterase inhibition), or indirect measures such as the degradation of sub-cellular lysosomes.

As with biomarkers of exposure, biomarkers of effect can be categorised as general or specific responses. General biomarkers include indicators of cellular and genetic damage such as increase of antioxidant enzyme activity, lysosomal damage, DNA damage and histopathological lesions (Table 6.1). Contaminant-specific biomarkers can be used when the mode of action of a chemical is known, such as inhibition of acetylcholinesterase by organophosphates and carbamates (Mayer *et al*., 1992).

Most effects biomarkers give general responses to a range of chemicals, which is useful as a screening tool in assessments when organisms in the environment are responding to exposure to a mixture of contaminants. Oxidative stress, for example, occurs in all living organisms when reactive oxygen species (or their by-products) cause cell and tissue injury. Exposure to a broad range of chemicals, including pesticides, metals and PAHs can increase the level of cellular oxidative stress in aquatic organisms (Winston, 1991; Livingstone, 2001; Banni *et al*., 2005; Lushchak, 2011). A breakdown in the antioxidant detoxification processes with the potential for higher order effects has been observed for metals in bivalves (Taylor and Maher, 2010, 2012a,b, 2014).

The division of biomarkers into categories of exposure or effect is to some extent arbitrary, since they are divided according to how they are used rather than by an inherent dichotomy (Suter, 2006). The responses of biomarkers can be seen as biological or biochemical effects indicating exposure to a stressor, which makes them theoretically useful as indicators of both exposure and effects (van der Oost *et al*., 2003). Biomarkers of exposure can be used to confirm and assess the exposure of individuals or populations to a particular substance group (for example metals, hydrocarbons, pesticides, etc.), providing a link between external exposure and internal dose. Biomarkers of effect can be used to document either pre-clinical alterations or adverse health effects due to external exposure and internal adsorption of a contaminant.

6.4 Commonly used biomarkers

Several biomarkers are now gaining relatively wide use and acceptance in ecological risk assessment programs throughout the world. The following section describes a selection from each of the categories of biomarkers currently in use (Table 6.1), in terms of their action in aquatic organisms and use in ecological risk assessment for sediment contaminants.

6.4.1 Biotransformation enzymes

Enhancement or inhibition of the activity of biotransformation enzymes is one of the most sensitive of the effects biomarkers (van der Oost *et al*., 2003). Biotransformation enzymes are classified as Phase I and Phase II enzymes according to the order in which they interact

Table 6.1. Common biomarkers of exposure and effect (modified from Taylor and Maher, 2010)

Category	Biomarker	Toxicant	Response	Examples of use
Biotransformation enzymes				
Phase I	Cytochrome P450	PAHs, PCBs, pesticides	+/–	Kim *et al.*, 2004a,b; Shaw *et al.*, 2004; Arun *et al.*, 2006; Ren *et al.*, 2014
	Ethoxyresorufin-O-deethylase (EROD)	PAHs, PCBs, pesticides	+	Fouchécourt *et al.*, 1999; Whyte *et al.*, 2000; Fossi *et al.*, 2004; Kirby *et al.*, 2004; Miller *et al.*, 2004
	Aryl hydrocarbon hydroxylase	PAHs, PCBs, pesticides	+	Bogovski *et al.*, 1998
Phase II	Glutathione S-transferase	Metals, PAHs, PCBs, pesticides	+/–	Bellas *et al.*, 2014; Regoli *et al.*, 2014; Ren *et al.*, 2014; Turja *et al.*, 2014; Vidal-Liñán *et al.*, 2014
Oxidative stress				
Enzymes	Glutathione peroxidase and reductase	Metals, PAHs, PCBs	+/–	Almeida *et al.*, 2004; Bellas *et al.*, 2014; Regoli *et al.*, 2014; Vidal-Liñán *et al.*, 2014
	Catalase/superoxide dismutase	Metals, PAHs, PCBs	+/–	van der Oost *et al.*, 2003; Company *et al.*, 2004; Caricato *et al.*, 2010; Regoli *et al.*, 2014
Cofactors	Total glutathione – reduced glutathione plus oxidised glutathione (GSH+ GSSG)	Metals, PAHs, PCBs, pesticides	+/–	Frenzilli *et al.*, 2004; Regoli *et al.*, 2004, 2014
	Ratio of reduced glutathione to oxidised glutathione (GSH:GSSG)	Metals, PAHs, PCBs	–	Cossu *et al.*, 2000; Hoffman, 2002; Tandon *et al.*, 2003; Maity *et al.*, 2008
Activity	Total antioxidant capacity	Metals, PAHs, PCBs	–	Moncheva *et al.*, 2004; Gorinstein *et al.*, 2005; Almeida *et al.*, 2007

Table 6.1. (Continued)

Category	Biomarker	Toxicant	Response	Examples of use
	Total oxygen scavenging capacity	Metals, PAHs, PCBs, pesticides	–	Regoli and Winston, 1999; Regoli, 2000; Regoli *et al.*, 2002; Regoli and Giuliani, 2014; Camus *et al.*, 2004
Damage	Lipid peroxidation	Metals, PAHs, PCBs, pesticides	+	Charissou *et al.*, 2004; Almeida *et al.*, 2007; Regoli *et al.*, 2014
Haematological	Aspartate and alanine aminotransferases	Metals, Cd, Cu, Hg	+	Benson *et al.*, 1988; Beyer *et al.*, 1996; Blasco and Puppo, 1999; de Aguiar *et al.*, 2004
	δ-aminolevulinic acid dehydratase	Metals, Pb, Zn	+	Rodriguez *et al.*, 1989; Burden *et al.*, 1998; Campana *et al.*, 2003; Perottoni *et al.*, 2005
Other proteins	Heat shock proteins	Heat, metals, PAHs	+	Cruz-Rodríguez and Chu, 2002; Feng *et al.*, 2003; Urani *et al.*, 2003; Bodin *et al.*, 2004
	Metallothioneins	Metals	+	Amiard *et al.*, 2006; Marie *et al.*, 2006; Caricato *et al.*, 2010; Regoli *et al.*, 2014
Neurotoxic	Acetylcholinesterase	Organophosphate and carbamate pesticides	–	Rickwood and Galloway, 2004; Caricato *et al.*, 2010; Bellas *et al.*, 2014; Regoli *et al.*, 2014; Vidal-Liñán *et al.*, 2014
Genotoxic	Micronuclei frequency	Metals, PAHs, PCBs	+	Bolognesi *et al.*, 2004; Galloway *et al.*, 2004b; Regoli *et al.*, 2014
	DNA strand breaks	Metals, PAHs, PCBs	+	Akcha *et al.*, 2004; Almeida *et al.*, 2007; Regoli *et al.*, 2014
	DNA adducts	Metals, PAHs, PCBs	+	Bodin *et al.*, 2004; Pisoni *et al.*, 2004; Almeida *et al.*, 2007

(continued)

Table 6.1. (Continued)

Category	Biomarker	Toxicant	Response	Examples of use
Cellular	Lysosomal stability	Metals, TiO_2 nanoparticles, other stressors	–	Galloway *et al.*, 2004b; Moore *et al.*, 2006; Caricato *et al.*, 2010; Balbi *et al.*, 2014; Turja *et al.*, 2014
Physiological	Histopathology	All xenobiotics	–	Farley, 1988; Sunila, 1988; Wedderburn *et al.*, 2000; Au, 2004; Zorita *et al.*, 2006
	Cellular energy allocation	All xenobiotics,	–	Smolders *et al.*, 2004; Cherkasov *et al.*, 2006
	Scope for growth	All xenobiotics	–	Smolders *et al.*, 2004; Burt *et al.*, 2007; Bellas *et al.*, 2014
	Condition index	All xenobiotics	–	Bodin *et al.*, 2004; Bellas *et al.*, 2014; Turja *et al.*, 2014
Reproductive	Vitellogenin	Dioxin, endosulfan, pesticides, metals	+	Houtman *et al.*, 2007; Dias *et al.*, 2014; Girish *et al.*, 2014; Prado *et al.*, 2014
	Gamete fertilisation	All xenobiotics	–	Ringwood and Conners, 2000; Ringwood *et al.*, 2004
	Embryo development	All xenobiotics	–	ASTM, 2012; Ringwood *et al.*, 2004; Balbi *et al.*, 2014
	Imposex	Tributyltin, Cu, other environmental stressors	+	Smith and McVeagh, 1991; Nias *et al.*, 1993; Galloway *et al.*, 2004b

+ = increased; – = decreased expression.

with substances to metabolise them. Phase I and Phase II biotransformation reactions usually work together in a sequential way to convert xenobiotics to more easily excreted metabolites. The major Phase I biotransformation enzyme is cytochrome P450 1A, which is involved in the initial phase of the metabolism of organic compounds such as hydrocarbons, pesticides and drugs (Snyder, 2000), largely through oxidative reactions (Buhler and Williams, 1988). The most commonly studied Phase II, or conjugating, enzymes are the glutathione S-transferases (GST), UDP-glucuronosyltransferases (UDPGT), and sulfotransferases (ST), which link metabolites to glutathione, glucuronic acid, and sulfate,

respectively (Buhler and Williams, 1988; Stegeman *et al*., 1992). The major pathway for electrophilic compounds and metabolites is conjugation with GST while the major route for nucleophilic compounds is glucuronic acid conjugation (George, 1994).

Compared to Phase I enzymes, the induction reaction of Phase II enzymes is generally less pronounced (George, 1994). They may be more useful in an integrated approach using a combination of biomarkers such as the biotransformation index (BTI, reflecting the ratio between Phase I and II activities), because this reflects a balance between bioactivation and detoxification (van der Oost *et al*., 1998).

Cytochrome P450 1A (also known as CYP1A) concentrations have been used to demonstrate that total PAHs in sediments led to impaired fish health in Puget Sound (Washington, USA), an urbanised estuary with limited circulation. Altered CYP1A activity has been related to increased PAHs in sediments and decreased health of English sole (*Pleuronectes vetulus*) (Myers *et al*., 2003; Johnson *et al*., 2008a). A dataset which has been built over decades has been successfully used to guide remediation and restoration efforts of several bays and waterways within Puget Sound (Johnson *et al*., 2008a) and the same biomarkers have been used to monitor the success of the remediation efforts. Decreases in biomarker responses were indicative of decreased bioavailable PAH concentrations following remediation (Myers *et al*., 2003; Johnson *et al*., 2008a).

Increased GST activity, correlated with both organic (aliphatic hydrocarbons, PCBs and PAHs) and metal contamination, has been demonstrated in several *in situ* monitoring programs using caged mussels *Mytilus galloprovincialis* (Serafim *et al*., 2011; Regoli *et al*., 2014; Vidal-Liñán *et al*., 2014) or clams *Ruditapes philippinarum* (Ramos-Gómez *et al*., 2011), and with wild mussel populations (Vidal-Liñán *et al*., 2010; Bellas *et al*., 2014). While GSTs are known for their role as primary catalysts in the conjugation of glutathione to organic electrophilic compounds, these enzymes also play a role in protection against oxidative stress by catalysing selenium-independent glutathione peroxidase activity (Manduzio *et al*., 2004). Correlations between GST induction in mussels and metal exposure have been reported in several studies (Canesi *et al*., 1999; Fernández *et al*., 2010b; Ciacci *et al*., 2012).

6.4.2 Oxidative stress biomarkers

Molecular oxygen is required by all aerobic organisms for the provision of energy through the coupling of oxidation to energy transfer via the phosphorylation of adenosine diphosphate (ADP). In aquatic organisms this process is managed by the mitochondrial electron transport system; in which oxygen undergoes a concerted four-electron reduction to water (Fig. 6.2) (Winston and Di Giulio, 1991). All aerobic life has the potential to experience oxidative stress when antioxidant defences are overwhelmed by activated oxygen species, also referred to as oxygen free-radicals, reactive oxygen species (ROS), reactive oxygen intermediates (ROIs) or oxyradicals (Winston, 1991). Changes in ROS may occur due to both contaminant and non-contaminant stress. Several exogenous compounds, particularly the Fenton-type metals iron and copper, can enhance intracellular oxyradical production through the process of redox cycling (Stegeman *et al*., 1992; van der Oost *et al*., 2003). Other redox inactive metals can indirectly increase ROS. For example, zinc and lead can deplete antioxidant capacity via inhibition of the glutathione reductase enzyme, while cadmium inhibits both the superoxide dismutase and catalase enzymes as well as reducing the scavenging potential of glutathione through binding to sulfhydryl groups (Ercal *et al*., 2001; Gazaryan *et al*., 2007; Regoli and Giuliani, 2014). Other chemicals which can increase the production of ROS, through the induction of the cytochrome P450 pathway, include

polycyclic aromatic hydrocarbons (PAHs), dioxin and dioxin-like chemicals, polychlorinated biphenyls (PCBs) and halogenated hydrocarbons (Regoli and Giuliani, 2014).

Biomarker measurements of the antioxidant system fall into four major categories:

- the enzymes superoxide dismutase and catalase which convert the superoxide anion (O_2^-) to hydrogen peroxide, and hydrogen peroxide to water and free oxygen, respectively (Fig. 6.2);
- the glutathione system including the glutathione peroxidase and reductase enzymes and their cofactor glutathione;
- the total capacity of the antioxidant system to neutralise reactive oxidant species; and
- damage such as lipid peroxidation.

Those biomarker measurements showing promise for use in sediment quality assessment are the total antioxidant capacity (TAOC) assay, which provides an overall measure of the ability of the ROS reduction system to neutralise reactive oxygen species, and lipid peroxidation which is a widely recognised consequence of oxyradical production (Winston and Di Giulio, 1991). These two biomarkers used in combination can provide evidence of both exposure and effects of contaminants.

Bacanskas *et al.* (2004) investigated differences in antioxidant defences between wild caught populations of killifish (*Fundulus heteroclitus*) from a creosote-contaminated inlet of the Elizabeth River, Virginia, USA, and reference killifish. Killifish at the site were found to exhibit chronic effects of exposure to creosote contaminated sediment but were relatively resistant to its acute effects, which exposure studies suggested may be associated with up-regulated antioxidant defences (Meyer *et al.*, 2003). The study identified differences in the antioxidant defence enzymes glutathione peroxidase and reductase and total glutathione, as well as in lipid peroxidation (a marker of oxidative stress), in populations with differing histories of contaminant response. These responses were also influenced by

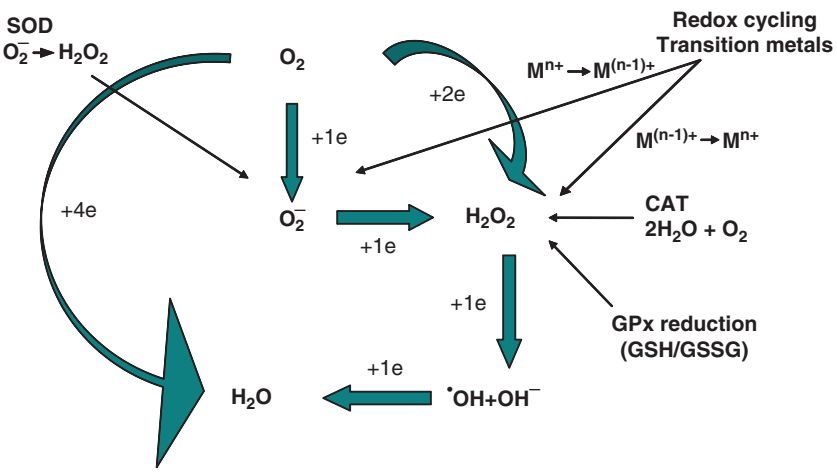

Figure 6.2. Oxygen reduction metabolism showing the 4-step electron-transfer reactions in the conversion of oxygen to water during energy transfer, and the interaction of redox-active metals in the process. The major reduction enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and the cycling of reduced and oxidised glutathione in the glutathione cycle (GSH/GSSG), which are responsible for the reduction of reactive oxygen species, are indicated at the steps where they are active (modified from Winston and Di Giulio, 1991). M stands for metal (that is, the redox cycling transition metals).

environmental conditions, including temperature, and by reproductive state; the authors therefore recommended taking care to identify and take into account these influences when interpreting contaminant effects (Bacanskas *et al*., 2004). It should be noted, however, that while these studies may indicate that some populations have the capacity to show elevated tolerance, it may also be the case that periodic low-level exposures that do not select for tolerant genotypes could contribute to persistent toxicity and long-term population effects (Ringwood and Conners, 2000; Ringwood *et al*., 2004).

Oxidative stress biomarkers were used to evaluate the persistence of PAHs from the *Prestige* oil spill, through their effects on the filter-feeding bivalve mussel *Mytilus edulis* (Fernández *et al*., 2010a). Mussels collected along a spatial gradient two years after the spill, when PAH concentrations in the area were returning to background levels, still showed some spill signature in their tissues. Although the PAH concentrations were low, an integrated oxidative stress response analysis showed the mussels had elevated oxidative stress but no changes in physiological condition. This suggested that oxidative stress biomarkers were a sensitive indicator showing that oxidative-stress-producing contaminants from the spill were still present in the environment (Fernández *et al*., 2010a).

6.4.3 Other protein biomarkers

Two commonly used protein biomarkers are the stress or heat shock proteins (hsp) and metallothioneins (MTs). In aquatic species, members of the hsp70 and hsp60 groups are highly conserved and exhibit measurable increases in synthesis in response to temperature and salinity (Werner, 2004), oxidative stress (Iwama *et al*., 1998), and environmental contaminants including metals (Bierkens *et al*., 1998; Boone and Vijayan, 2002; Aït-Aïssa *et al*., 2003; Feng *et al*., 2003). In particular, hsp72 is only synthesised in response to environmental stressors and is not found in most cells under normal conditions, making it an excellent candidate as an exposure biomarker for chemical contamination (Stegeman *et al*., 1992). Metallothionein is a low molecular weight (≤10 kDa) cysteine-rich metal-binding protein synthesised in response to metal exposure (Roesijadi, 1996), which may have potential as a biomarker of exposure to toxic metals (Garvey, 1990; Petering *et al*., 1990; Sanders, 1990; Stegeman *et al*., 1992; van der Oost *et al*., 2003).

Amiard *et al*. (2006) reviewed the use of metallothioneins as biomarkers of metal contamination, in three groups of aquatic invertebrates routinely used for risk assessment: molluscs, crustaceans and annelid worms. They focused particularly on the intraspecific and interspecific variability in the expression of metallothioneins which can be influenced by both physico-chemical and physiological conditions. They concluded that despite the inherent variability, metallothioneins do play a role in routine handling of essential metals zinc and copper and in the detoxification of these metals in excess, intracellularly, and of non-essential metals including Cd, Ag and Hg. It was recommended that metallothioneins would make useful response biomarkers in well-designed risk assessment sampling programs in an integrated response index using a suite of biomarker measurements.

The use of heat shock proteins (hsp) as biomarkers of biotic and abiotic stressors has been reviewed for fish (Iwama *et al*., 1998) and other aquatic organisms (Sanders, 1993). The hsp response has been shown to be related to both the sensing of natural and anthropogenic stressors and subsequent cellular effects which may adapt the cell to cope with the stressor. They are, therefore, considered to have a potential role in risk assessment as a first-order biomarker of general stress response, reflecting an integrated organism stress load regardless of the number of stressors present (Sanders, 1990). As with metallothioneins, the use of hsp within a suite of biomarkers and the application of an integrated

response index may enable a specific contaminant to be linked to the hsp stress response. This approach has been successfully applied by Werner *et al*. (2004); they measured several biomarker responses including hsp70, lysosomal membrane damage and histopathological lesions in the sediment-dwelling deposit feeding bivalve clam *Macoma nasuta* exposed to sediments from San Francisco Bay contaminated with metals, PAHs and the organochlorine pesticides aldrin, *p,p′*-DDT and its metabolites *p,p′*-DDD and *p,p′*-DDE. Correlation analysis linked tissue concentrations of DDT and its metabolites with increased hsp70 expression but not with any of the other contaminants present.

Proteomics biomarkers have recently been introduced to the field of ecotoxicology. These experiments are typically conducted by visualising the 3000 or so most abundant proteins in the cytosolic fraction via 2-D gel electrophoresis, and comparing the relative abundance of each spot (peptide). Studies by Costa *et al*. (2012) with the proteome of Senegalese sole (*Solea senegalensis*) exposed to contaminated sediments in the laboratory and in a contaminated harbour, and by Thompson *et al*. (2012) with the proteome of the Sydney rock oyster (*Saccostrea glomerata*) exposed to metals in Lake Macquarie, New South Wales (NSW), Australia, demonstrate how this approach has been used to determine protein abundance. In both studies, some proteins with different abundances associated with contaminant exposure were identified. In both cases, a small number of the differentially expressed proteins had known stress-response functions, while the majority were identified as cytoskeletal proteins.

6.4.4 Neurotoxic damage biomarkers

The principal neurotoxic enzyme identified in aquatic organisms is acetylcholinesterase (AChE), which is involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings that are vital for normal sensory and neuromuscular function. Activity of AChE is inhibited by organophosphate and carbamate pesticides, and it has been used in fish studies as an exposure biomarker for these xenobiotics (de Aguiar *et al*., 2004; Eder *et al*., 2004). In mussels it was not found to be a reliable indicator of organophosphate exposure (Cajaraville *et al*., 2000; Rickwood and Galloway, 2004), but was a sensitive measure for PAH exposure (Vidal-Liñán *et al*., 2014). Measurements in the polychaete worm *Nereis diversicolor* showed it to be a sensitive biomarker of PCB exposure (Durou *et al*., 2007). Solé *et al*. (2009) used a series of biomarkers in the ragworm *Nereis diversicolor* and the clam *Scrobicularia plana* to identify agricultural pollution sources and assess the recovery of an estuarine system following the installation of a sewage treatment plant and cessation of raw sewage discharge. The study found that AChE inhibition in ragworms was a sensitive marker, demonstrating changes in inputs of neurotoxic contaminants with time.

6.4.5 Cellular damage biomarkers

Cell damage may be used as a biomarker of both exposure to and effect of environmental contaminants. A widely used general biomarker of effect is measured by the lysosomal membrane stability assay. While the lysosomal membrane may be affected by a range of stressors it is particularly sensitive to metal toxicity. Lysosomes are important in sequestration and detoxification of metals. High metal concentrations may induce synthesis of metallothioneins which enter lysosomes for degradation and normal protein turnover, thus overloading their storage and detoxification capacity (Viarengo, 1989). Destabilisation of the lysosomal membrane following exposure to metals may result from direct interaction of metals with the lysosomal membrane and from metal-induced oxidative stress (Regoli *et al*., 1998). Lysosome membrane destabilisation has proved a useful ‘effect

biomarker' of metal exposure in both field and laboratory (Regoli *et al*., 1998; Ringwood *et al*., 2002; Taylor and Maher, 2012a, 2014).

Edge *et al*. (2012) used lysosomal membrane stability as a cellular biomarker to assess responses of the Sydney rock oyster *Saccostrea glomerata* to both metal and petroleum-derived sediment contaminants in estuaries of coastal NSW. The study found lysosomal membrane stability was more predictive of environmental stress and estuary sediment contamination than the oxidative stress biomarkers glutathione (GSH) and lipid peroxidation. Lysosomal membrane stability also correlated well with reproductive endpoints including fertilisation and embryo development.

Changes, at the molecular level, in the expression of the various enzymes involved in the conjugation, detoxification and excretion of toxins, are sensitive first-order measures of exposure and, in some instances, of effect. Also, measures of cell integrity and tissue morphology offer a second-order measure of exposure and more particularly of effect. Being at a higher order of biological organisation, the cellular and tissue effect response is less likely to be specific for a particular contaminant but rather a general response often indicative of chronic toxicity (Bayne *et al*., 1985; van der Oost *et al*., 2003).

6.4.6 Genotoxic damage biomarkers

Two commonly used genotoxic damage biomarker assays are the micronucleus and 'comet' assays. As an index of chromosomal damage the micronucleus test is based on the enumeration of downstream aberrations after DNA damage and shows a time-integrated response to contaminants. An advantage is that it is a fast and sensitive test for detection of genomic damage due to both clastogenic effects and alterations of the mitotic spindle (Migliore *et al*., 1987). The micronucleus test has proved suitable for application to aquatic invertebrates and is simple and rapid to perform (Bolognesi *et al*., 2004).

DNA damage can also be measured using the 'comet' assay, which uses a denaturing buffer to 'unwind' DNA. The fragmented portions of the genome are pulled behind the cell during electrophoresis, causing a cell with a high proportion of strand breaks to have an appearance similar to a comet. This approach has been used to assess the genotoxic potential of sediments in San Diego Harbor in California (USA) to deployed and resident mussels (*Mytilus edulis*) by Steinert *et al*. (1998) who found DNA strand breaks in deployed mussels were well correlated with levels of sediment contaminants, notably Hg, Cu and Zn. In grass shrimp (*Palaemonetes pugio*) collected along an estuarine gradient of PAH contamination, increased incidence of breaks in DNA strands and decreased production and hatching rates of embryos were demonstrated to be correlated with total PAHs in the sediment, (Lee *et al*., 2004).

Cheung *et al*. (2006) used the comet assay to assess the sensitivity of two bivalve molluscs – the sediment-dwelling cockle *Cerastoderma edule* and the filter-feeding mussel *Mytilus edulis* – to the reactive oxygen species hydrogen peroxide (H_2O_2). Increases in DNA strand breaks were found to be significantly concentration-dependent for both bivalves, but the cockle had the greater sensitivity to H_2O_2-induced oxidative damage. This demonstrates its potential as a bioindicator species for risk assessment of sediments.

DNA adducts occur when pieces of DNA become covalently bonded to exogenous chemicals, and are usually a biomarker of exposure to carcinogens. Bocquené *et al*. (2004) measured DNA adducts over three years as part of a suite of biomarkers specific to PAHs, to assess recovery of mussels (*Mytilus edulis*) following the wreck of the *Erika* oil tanker. DNA adducts were elevated in affected sites in the six months following the spill, but then returned to background levels. An integrated biomarker response index was developed as

part of a weight-of-evidence assessment of the spill site which indicated that mussels were only affected in the first year after the spill. The survey confirmed the measurement of DNA adducts as a reliable biomarker of PAH exposure and its effects on mussels, as it is a direct end product of their metabolism and less subject to biological variation than the enzymes that manage metabolism.

6.4.7 Physiological biomarkers

Physiological biomarkers can be used as indicators of both exposure to and effect of xenobiotics and may be useful in integrating the effects of several stressors by quantifying organism health (Mayer *et al*., 1992). A widely used condition index measure for bivalves is based on the measurement of the ratio of soft tissue weight to valve (shell) weight. A high ratio indicates good physiological condition and reduced ratios indicate poor physiological condition. It should be noted that condition index can also be influenced by natural stressors including temperature, salinity, dissolved oxygen and food availability.

An optimal assessment of the altered health of an organism or ecosystem will use a range of indicators selected from different levels of biological organisation. While measurements at the molecular and cellular level provide the greatest sensitivity, the overall fitness can be better assessed using physiological responses because these represent an integration of individual enzymatic and cellular effects (Duquesne *et al*., 2004).

Boldina-Cosqueric *et al*. (2010) used a multi-level biomarker approach with the endobenthic clams *Scrobicularia plana* from three estuaries along a contamination gradient in France. Biomarkers of exposure and effects at the subcellular level, including metallothionein induction, GST activity and AChE inhibition, correlated well with the contamination gradient, while changes in physiological biomarkers including glycogen reserves, water content, total lipids and gonadal–somatic index did not differ among estuaries. Changes in burrowing behaviour, however, thought to be related to physiological impairment, were correlated with the contaminant gradient, and clams from the most contaminated estuary were the smallest. The multi-level biomarker approach has also been applied by Kumar *et al*. (2010) who exposed the freshwater shrimp *Paratya australiensis* to the organophosphate insecticide profenofos, and demonstrated links between AChE inhibition and reduced physiological behaviour measured as chemotaxis responses such as approaching and grasping a chemo-attractant source.

6.4.8 Reproductive biomarkers

Linking biomarkers at the sub-organism level to life-history characteristics, such as reproduction and survival, shows that biomarkers are effective for toxicity assessment and predicting community-level effects. Tests of reproductive success in the oyster *Crassostrea virginica* have demonstrated links both to enzymatic biomarkers (depletion of gonadal GSH; Ringwood and Conners, 2000) and to cellular biomarkers (lysosomal membrane destabilisation; Ringwood *et al*., 2004). The tests examine both gamete fertilisation and embryonic development of adult oysters exposed to contaminant. The tests can also be used to examine successful fertilisation and the development of the embryos themselves, during exposure to a variety of contaminant mixtures, reflecting potential contaminant effects on sperm–egg interaction and development processes. Oyster sperm and eggs are mixed in seawater and incubated for 2 h, and the evaluation of gamete fertilisation looks for successful cleavage of the eggs. Fertilised eggs incubated for 48 h can be assessed for embryonic development. After this time, normal embryos have typically reached the veliger larval stage characterised by a D-shaped shell; whereas abnormal embryos do not develop a shell, or develop an abnormal shell, or may arrest in earlier stages (Ringwood and Conners, 2000).

Imposex, the imposition of male sex organs including a penis and vas deferens, has been observed in marine gastropods exposed to tributyltin (TBT) (Bryan *et al*., 1986; Smith and McVeagh, 1991). Other chemicals may also disrupt endocrine function in gastropods: copper and other environmental stressors, for example, induced imposex in *Lepsiella vinosa* (Nias *et al*., 1993).

Levels of reproductive hormones have been shown to be altered and reproductive function impaired in response to a range of chemical stressors, both organic and inorganic (Chen, 1988; Thomas, 1988; Depledge and Billinghurst, 1999; Siah *et al*., 2003). Vitellogenin is a precursor protein of egg yolk which is released into the blood stream and sequestered in the developing oocyte; it is a commonly used reproductive biomarker. Impaired reproductive function in fish, associated with altered vitellogenin levels, has been induced in rainbow trout by cadmium (Haux *et al*., 1988), and in white sucker (*Catostomus commersoni*) (McMaster *et al*., 1991) and Florida largemouth bass (*Micropterus salmoides floridanus*) by estrogenic compounds associated with bleached kraft mill effluent (Sepúlveda *et al*., 2002). A method for detecting the presence of vitellogenin in the blood of male mosquitofish (*Gambusia affinis*), which is not detectable normally, has been developed as a biomarker of estrogenic exposure by Tolar *et al*. (2001). In areas of Puget Sound, north-west USA, elevated levels of vitellogenin have been found in male English sole (Johnson *et al*., 2008b). Although the levels of vitellogenin did not correlate with histological changes in the ovary or testes, they were associated with alterations in the timing of spawning. The sources of xenoestrogens could not be identified in this study, but it is well known that estrogenic alkylphenol ethoxylates, surfactants found in many common detergents, are discharged with sewage and accumulate in sediments (Ferguson *et al*., 2001).

6.5 Biomarker selection

As with other aspects of study design, selection of a biomarker depends on the question to be answered. Biological responses, and therefore biomarker choice, also depend on the mode of action of the contaminant of interest and the level of biological organisation being examined. It is necessary to determine whether the study requires biomarkers of exposure to a chemical or group of chemicals, or a biomarker of toxic effect, or whether a combination of these is preferable.

To use biomarkers as a line of evidence, the following basic criteria have been proposed (Huggett *et al*., 1992; Svendsen *et al*., 2004; Hook *et al*., 2014).

1. A clear dose–response relationship should be demonstrated for target contaminants, noting that the cause of response may include multiple stressors.
2. The sensitivity of the biomarker to the contaminant should ideally be greater than the sensitivity of growth and reproduction to the same contaminant.
3. Ecological relevance should be demonstrated (that is, effects that influence an organism's ecological competitiveness through a strong influence on survival, growth and reproductive ability).
4. The effects of confounding non-chemical factors, such as season, temperature or size, should be understood.
5. Some knowledge of the chemical specificity is desirable to distinguish between non-specific biomarkers responding to a wide range of contaminants and those that are more specific to particular contaminants or contaminant classes.
6. The applicability of the biomarker for the chosen test species needs to have been demonstrated.

7. Time–response relationships for the biomarker need to be understood (how soon after exposure is the response seen and how long after exposure does the response persist).
8. There should be no methodological concerns such as reproducibility, robustness, ease of use.
9. There needs to be broad public, industry and regulator confidence in and acceptance of the biomarker for routine application in assessment.

It should be noted that these criteria have been proposed as the ideal for the selection and use of biomarkers in assessment. Some of these may be difficult to meet and so may serve to impede the use of biomarkers. These criteria should be seen as a guide and target for a program using biomarkers. They are relevant to the selection and use of biomarkers in general, but individual biomarkers will not fulfil all of these basic criteria. None of the biomarkers of effect or exposure, which are analysed at the biochemical or physiological level, have been found applicable also at the population level, let alone the ecosystem level, so most biomarkers would fail the ecological relevance criterion. Many biomarkers can be seen as indications of decreased health of individuals, which would ultimately affect the health of populations and systems.

Coupling classes of contaminant-specific exposure biomarkers with effects biomarkers in a single assessment could both predict overall organism health and identify causative agents (Fig. 6.2). Although that combination would address neither the reversibility of deleterious effects if the stressor were to be removed, nor the capacity for organisms to avoid the stressors, it would still provide insight for a weight-of-evidence environmental risk assessment.

6.6 Use of multiple biomarkers

To understand biological or ecological effects of exposure to chemical and other environmental stressors it is necessary to establish relationships between chemical contaminant concentrations in the sediment (exposure), organism tissues (dose) and biological effects (response), based on toxic modes of action of the contaminants (Fig. 6.3). In sediment quality assessments, biomarker effects on benthic organisms can serve as an additional line of evidence as part of a weight-of-evidence assessment; biomarker effects are particularly useful as early measures of response when examining contaminant exposure–dose–response relationships.

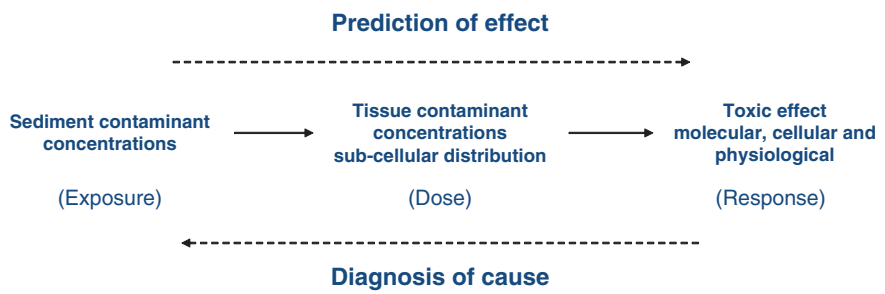

Figure 6.3. Ecotoxicological framework allowing prediction and diagnosis of effects from exposure to contaminants (modified from Widdows and Donkin, 1992).

Commonly there are two types of sediment risk assessment: those where a single contaminant class, for example metals or organics, is present; and those involving multiple contaminant types and effects. In both cases, the exposure–dose–response framework can be used, although different specific biomarkers will be needed to investigate specific contaminants of concern and their effects.

6.6.1 Single contaminant classes

For single contaminant classes, for example metals, a suite of linked biomarkers can be used to give comprehensive information on mechanisms and effects at various biochemical levels. Often an improved inference can be obtained through the use of a suite of related biomarkers.

The oxidative system offers a range of general response and effect biomarkers which have been shown to be sensitive to metals through perturbations in the redox cycle and other oxidative pathways (Fig. 6.2). The measurement of a suite of biomarkers within this system, from reactive oxygen species, through catalysing and Phase II enzymes, to oxidative damage indices, is valuable for assessing molecular-level exposure and effects. Lysosomes are involved in metal management and are also susceptible to oxidative damage, and therefore measurement of their integrity offers a useful biomarker of effect at the cellular level. Impairment of the lysosomal membrane can be considered a second-order effect that would follow perturbations at the molecular level. The frequency of micronuclei occurrence offers a measure of DNA damage; it may aid in completing a suite of interrelated reactions resulting from exposure of individual organisms to toxic concentrations of metals.

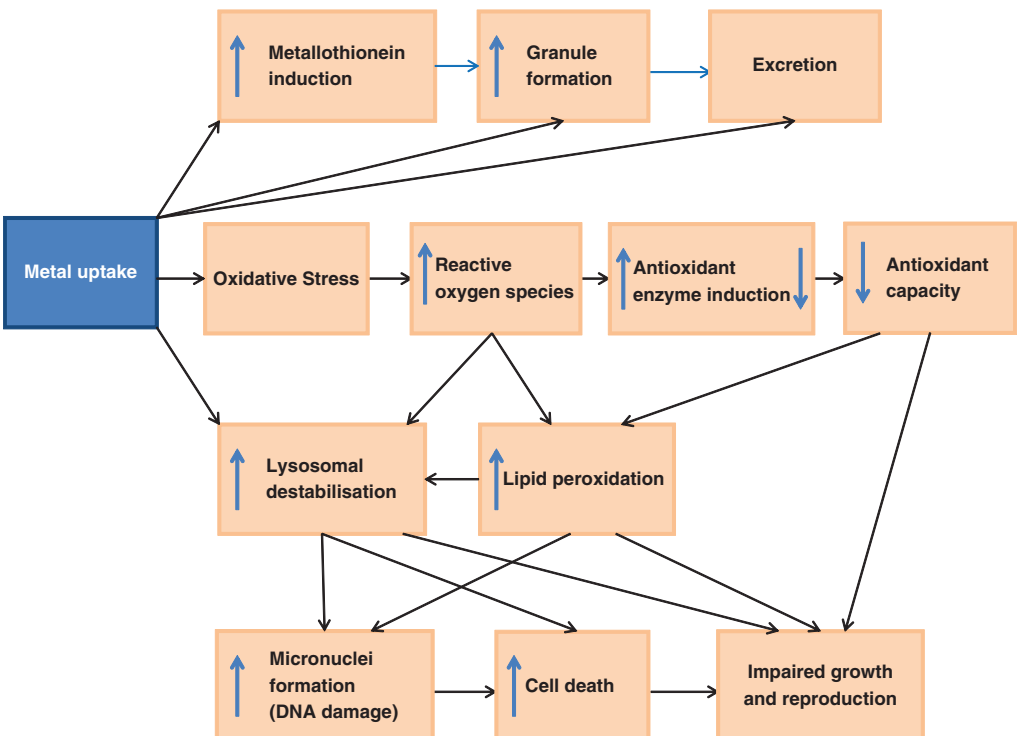

Figure 6.4. Flow diagram demonstrating a possible cascade of interlinked cellular reactions which may occur in response to metal exposure and could guide selection of potential biomarkers.

Linking this to organism physiological condition, using a 'measure of condition' index, gives an indication of the potential for these effects to result in reproductive and ultimately population-level effects. An inter-related suite of biomarker measurements is necessary for interpreting the molecular perturbations measured and therefore linking them to potential higher order effects (Fig. 6.4). By identifying and establishing these relationships at these levels of biological organisation, it is possible to improve understanding of the mechanisms of stress responses in ecological systems. Ultimately that could result in improved predictive capability of ecological risk assessment and also allow for more informed decisions regarding remedial actions.

Models such as that illustrated in Fig. 6.4 do not encompass all possible scenarios but are proposed as an approach to selecting relevant biomarkers for specific assessments.

6.6.2 Multiple contaminants

Risk assessment of multiple contaminants, applying a weight-of-evidence approach, uses a suite of biomarkers, encompassing measures of molecular damage, development abnormalities and physiological impairment (Galloway *et al*., 2004a). Typically, biomarkers are chosen from the eight classes of biomarkers described in Section 6.4 above. In addition, when a particular contaminant of interest is expected to be present, a specific biomarker of effect is included. If, for example, tributyltin is an expected contaminant, imposex measurements are included, while EROD measurements would be used where pyrogenic hydrocarbons are anticipated (Johnson *et al*., 2008a).

Responses for all biomarkers are analysed together, using multivariate techniques such as multi-dimensional scaling (MDS) which summarises patterns of variation of different variables. With this technique, sites can be classified relative to uncontaminated reference locations or a gradient of contamination, based on effects (see Section 6.10.2 below). Combined with bioaccumulation measurements, this approach can identify the contaminants causing effects.

6.7 Linking biomarkers and population effects

Before biomarkers can become an integral part of risk assessment programs, they need to be usable for predicting effects at population or community levels. At present, measurements of lysosomal stability can be directly linked to reproductive success in molluscs (Ringwood and Conners, 2000; Ringwood *et al*., 2004) to provide some prediction of population effects. Similarly, physiological measurements, such as of cellular energy allocation, are being developed to predict population effects (De Coen and Janssen, 2003; Smolders *et al*., 2004; Moolman *et al*., 2007). Models are being used to extrapolate data from individuals to field populations (Hutchinson *et al*., 2006). Vitellogenin-induction in males, for example, has been used to predict decreased growth rates in fish populations exposed to estradiol (Gleason and Nacci, 2001).

6.8 Quality assurance

Hutchinson *et al*. (2006) stressed that for biomarkers to fulfil their potential in risk assessment they should be mechanistically relevant and reproducible. As progress is made in the use of biomarkers in risk assessment, it will be necessary to evaluate the variability in the different biomarker responses and adverse-effect endpoints, as is normal practice in other areas of ecotoxicology and mammalian toxicology. The OECD (2005) guidance document on the validation of test methods defines the following terms in inter-assay comparability. (The final point is from Hutchinson *et al*., 2006).

1. *Relevance*: the description of the relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect and species of interest.
2. *Reliability*: the extent of reproducibility of results from a test over time within and among laboratories when performed using the same protocol.
3. *Reproducibility*: the agreement among results obtained from testing the same substance using the same test protocol (see 'Reliability').
4. *Repeatability*: the agreement among test results obtained within a single laboratory when the procedure is performed on the same substance under identical conditions.
5. *Robustness*: the insensitivity of a test to departure from the specified test conditions; the ability of a test to provide similar results over a range of test conditions under which the test may be used in different laboratories.
6. *Transferability*: the ability of a test method or procedure to be accurately and reliably performed in independent, competent laboratories.
7. *Comparability*: the comparison of results from laboratories measuring the same endpoint using different protocols.

6.9 Choice of organisms

The organisms chosen to measure biomarkers should satisfy the criteria outlined in Chapter 5 Section 5.3 for use in bioaccumulation studies. Biomarkers may also vary with temperature, lipid content, sex, age and reproductive state (Bodin *et al.*, 2004; Hagger *et al.*, 2006; Caricato *et al.*, 2010) and these factors will need to be considered to minimise variability and aid in interpretation of results. The capacity to differentiate between statistically detectable versus biologically significant responses is also required. Similar to acceptability criteria for toxicity tests, results should be both significantly different to controls or reference organisms and differ by a specified margin. For example, it appeared that a degree of lysosomal membrane destabilisation of ~60% was necessary to represent an environmentally significant level of effect to the oyster *Saccostrea glomerata*, in the study by Edge *et al.* (2014). In addition, some biomarkers are not measured using whole tissues but instead use specific tissues or fluids such as digestive system, ovaries, haemolymph or urine (Watson, 2004; Houtman *et al.*, 2007; Caricato *et al.*, 2010), and analyses will require organisms from which sufficient tissue or fluid can be obtained.

6.10 Study design and statistical analysis

As with the measurement of bioaccumulation, biomarker measurements can be made using field or laboratory exposures, using either passive biomonitoring of indigenous organisms or active biomonitoring of field- or laboratory-transplanted organisms (see Chapter 5 Section 5.4). Usually, the same organisms that have been used to measure bioaccumulation are suitable for measuring biomarkers.

6.10.1 Single contaminant classes

The three approaches for a single contaminant class are illustrated in a study using the bivalve or cockle *Anadara trapezia* to measure the responses of four biomarkers – total antioxidant capacity (TAOC), lipid peroxidation, lysosomal destabilisation, and micronuclei frequency – to metals accumulated from contaminated sediments. The study focused on Lake Macquarie, NSW, Australia (for a map, see Chapter 5, Fig. 5.2). Protocols for

measuring these biomarkers are provided in Appendix J. The experimental designs used are the same as those outlined in Chapter 5 Section 5.4.2 and the biomarker analysis complements the lead (Pb) bioaccumulation results reported there. The lead concentrations reported in those studies affect the cockle through *exposure*, while the bioaccumulated metals in those studies are the *dose* driving the change in *responses* (TAOC, lipid peroxidation, lysosomal destabilisation and micronuclei frequency).

Passive sampling

Results and analysis

Indigenous *Anadara trapezia* collected from Cockle Bay in Lake Macquarie (Chapter 5, Fig. 5.2) had accumulated a tissue lead dose similar to those accumulated by organisms transplanted into Cockle Bay sediments in the field and laboratory (Chapter 5, Figs 5.4, 5.6 and 5.8). In both field- and laboratory-transplanted *A. trapezia*, the oxidative stress biomarker TAOC and oxidative damage biomarker lipid peroxidation were at similar levels, and indicated impairment when compared to reference organisms (Figs 6.5 and 6.6). Indigenous *A. trapezia* and reference organisms showed similar percentages of lysosomal destabilisation, which were lower than those in field- and laboratory-transplanted *A. trapezia* which had no previous history of metal exposure (Fig. 6.7). These results suggest that chronic lead exposure over generations in Cockle Bay has produced an indigenous population with some metal tolerance. When designing assessment studies,

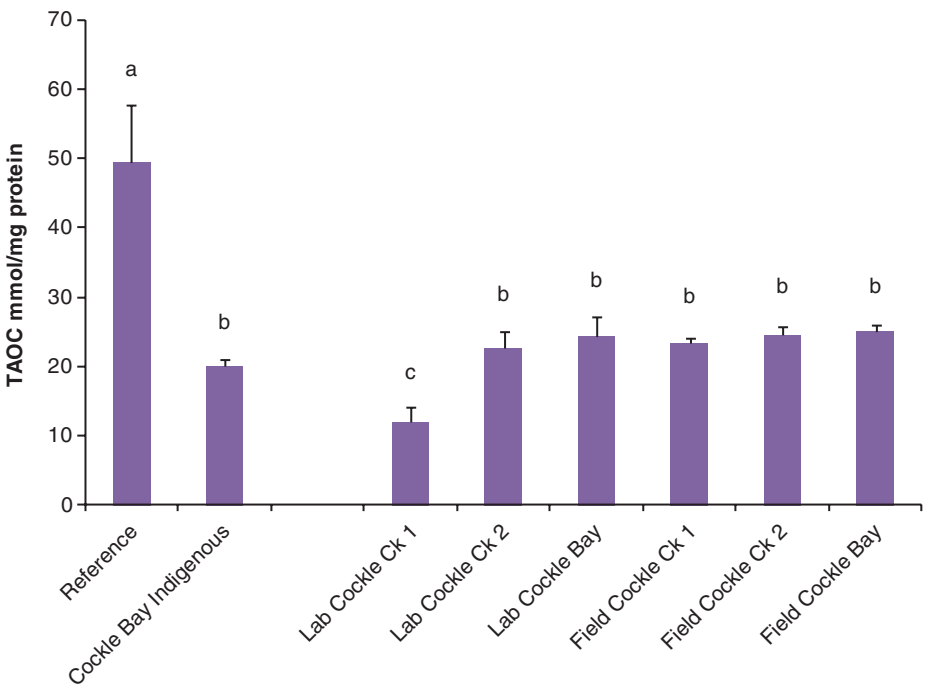

Figure 6.5. Oxidative stress biomarker, total antioxidant capacity (TAOC), measured in *Anadara trapezia*: indigenous, field-transplanted or laboratory-transplanted into sediments from a lead contamination gradient in Lake Macquarie, NSW, Australia. Different letters denote significant differences ($P < 0.05$).

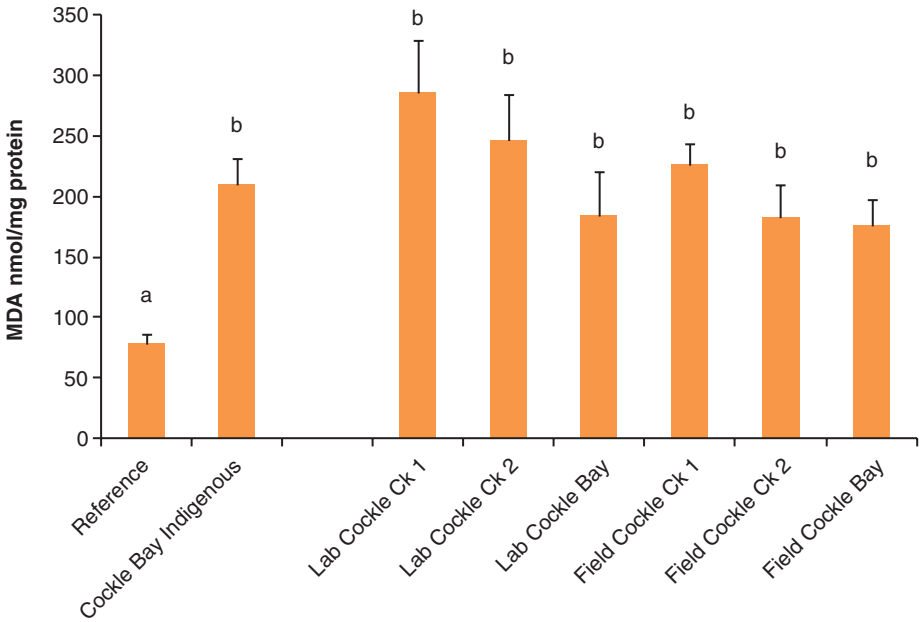

Figure 6.6. Lipid peroxidation, an oxidative stress damage biomarker, measured as malondialdehyde (MDA) in *Anadara trapezia*: indigenous, field-transplanted or laboratory-transplanted into sediments from a lead contamination gradient in Lake Macquarie, NSW, Australia. Different letters denote significant differences ($P < 0.05$).

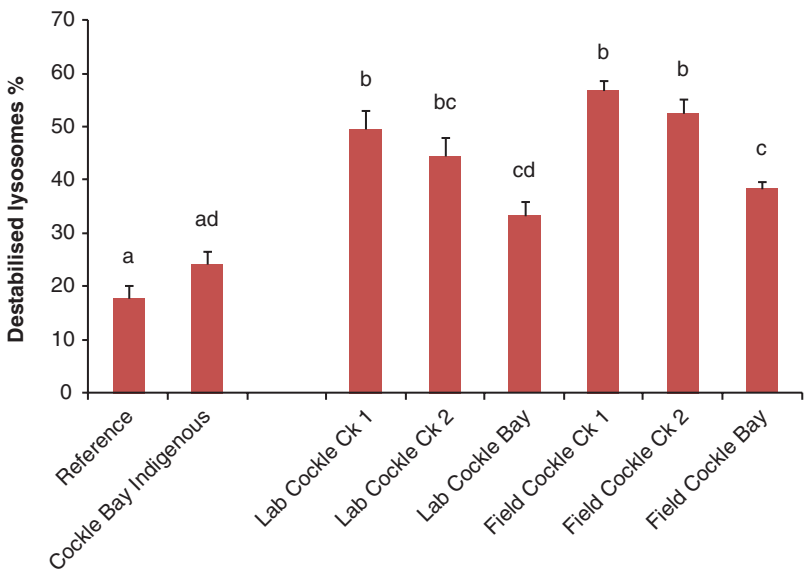

Figure 6.7. Lysosomal destabilisation, a cellular biomarker, measured in *Anadara trapezia*: indigenous, field-transplanted or laboratory-transplanted into sediments from a lead contamination gradient in Lake Macquarie, NSW, Australia. Different letters denote significant differences ($P < 0.05$).

consideration needs to be given to the potential for differences in tolerance to exist between indigenous populations and those with no previous exposure history.

Field-transplantation

Study results and analysis

Lead accumulation in the field-transplanted *A. trapezia* reflected the sediment exposure concentrations (Chapter 5, Figs 5.3 and 5.6). While there was little difference in the oxidative stress biomarker TAOC (Fig. 6.5), there was a dose-dependent increase in the oxidative damage biomarker – lipid peroxidation – and in the cellular biomarker – lysosome destabilisation (Figs 6.6 and 6.7). These results demonstrate the value of using a suite of interrelated biomarkers to gain a representative picture of organism toxicity.

Laboratory transplantation – microcosm exposure

Study results and analysis

Anadara trapezia exposed to a lead gradient in Lake Macquarie sediments, from Cockle Creek to Cockle Bay (Chapter 5, Fig. 5.2) accumulated higher tissue lead doses at the highest exposure, and similar tissue concentrations at the lower exposures, which reflect the sediment exposure concentrations (Chapter 5, Figs 5.3 and 5.8). When exposed in laboratory microcosms, the organisms at the highest lead exposure accumulated a higher tissue lead dose compared to field-transplanted organisms (Chapter 5, Figs 5.6 and 5.8). This was reflected in the lower TAOC and higher lipid peroxidation measured in organisms from this treatment (Figs 6.5 and 6.6). As lead exposure and tissue dose increased, lipid peroxidation, lysosomal destabilisation and micronuclei frequency all increased in a dose-dependent manner across exposure treatments (Figs 6.6, 6.7 and 6.8). These responses, measured in an exposure–dose framework, clearly demonstrated impairment of organism

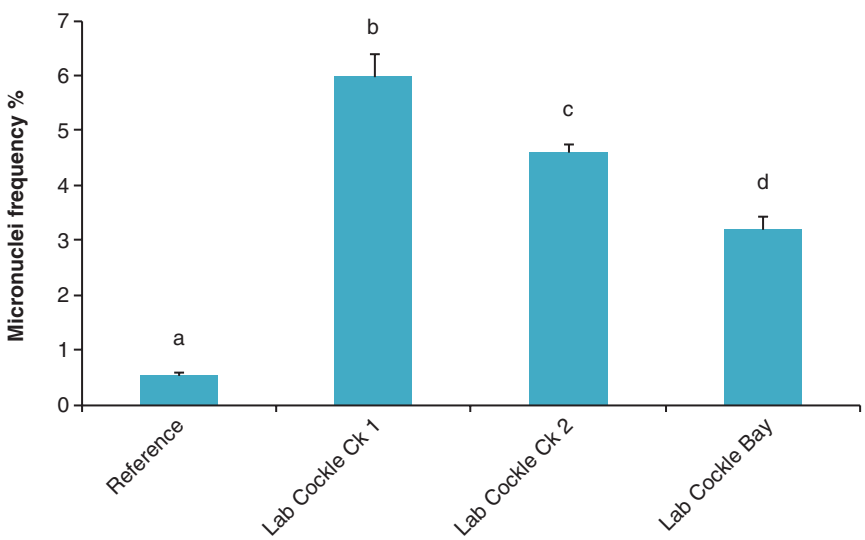

Figure 6.8. Genotoxic biomarker, micronuclei frequency, measured in *Anadara trapezia* laboratory-exposed to sediments from a lead contamination gradient in Lake Macquarie, NSW, Australia. Different letters denote significant differences ($P < 0.05$).

health at enzymatic, cellular and genotoxic levels of biological organisation which may have the potential to translate into population and ecosystem effects.

6.10.2 Multiple contaminants

Sydney Harbour, in NSW, Australia, has an extensive history of industrial development leading to a mixture of contaminants in its sediments, notably: polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Birch *et al*., 2007; Roach *et al*., 2009b).

The following example illustrates a biomarker approach for assessing multiple contaminants. Data for this example were provided by the NSW Office of Environment and Heritage (K. Edge, personal communication).

The indigenous smooth toadfish *Tetractenos glaber* is considered a relevant biomonitoring species because it is endemic to Australian estuaries and has a distribution spanning the coastline from southern Queensland, through NSW and Victoria to the central South Australian coast and Tasmania. The species is a site-specific resident benthic-feeding carnivorous fish up to 120 mm in length, with a diet consisting of molluscs and crabs (Booth and Schultz, 1999).

Toadfish *T. glaber* were collected from four locations along an organic contamination gradient extending from Homebush Bay (HB), through Five Dock Bay (FD), Shell Cove (SC) to Roseville Chase (RC) (all heavily modified and contaminated sites) within Sydney Harbour, NSW (Birch *et al*., 2007; Roach *et al*., 2009a), and compared with *T. glaber* from two reference locations within Port Hacking (PH) and the Hawkesbury River (Berowra)

Figure 6.9. Sampling locations along a contamination gradient in Sydney Harbour, and reference locations at Berowra Waters and Port Hacking, NSW, Australia.

(BW) (which are relatively unmodified sites) (Fig. 6.9). Six to ten adult male fish were sampled from each of the sites, using baited traps.

An integrated biomarker approach was used to assess the response of *T. glaber* to the organic contamination gradient and establish the sites of greatest concern. The Phase I bioactivation biomarker ethoxyresorufin O-deethylase (EROD), and Phase II conjugating detoxification biomarker glutathione S-transferase (GST), were combined with a damage biomarker (lipid peroxidation) and a measure of reproductive function (gonadal histopathology) to assess the response to organic contaminants.

The EROD activity, which is a reliable indicator of exposure to organic pollutants in fish (Gagnon and Holdway, 2002), was quantified using a modified version of the fluorescence method of Burke and Mayer (1974). Briefly, the substrate 7-ethoxyresorufin is metabolised to its first intermediate product, resorufin, which is then measured on a fluorescence spectrophotometer at excitation and emission wavelengths of 530 nm and 585 nm. The GST activity was quantified using the Luminos fluorescence-based assay kit (#K008-F1; Luminos, Ann Arbor, MI, USA), and lipid peroxidation was assessed by measuring thiobarbituric acid reactive substances (TBARS). The results are presented in relation to an enzyme unit (U) that represents the amount of a particular enzyme (for example mU/mL). Male gonadal sections were examined for the presence of testicular oocytes (testis-ova), altered spermatogenesis and increased testicular degeneration, as described in the OECD *Guidance document for the diagnosis of endocrine-related histopathology of fish gonads* (Johnson *et al.*, 2009). Transverse sections from the anterior, mid- and posterior parts of each gonad were examined. Excised gonad samples were fixed in Bouin's solution dehydrated in an ethanol series, cleared in histolene, and embedded in paraffin wax. Duplicate 5 µm sections were mounted on slides, stained with either haematoxylin and eosin or Mallory's triple stain, and examined by light microscopy. Identification of the reproductive abnormalities (pigmented cell accumulations, germ cell syncytia, interstitial fibrosis and cell vacuolisation) was based on descriptions in Takashima and Hibiya (1995), Dietrich and Krieger (2009) and Genten *et al.* (2009) (Fig. 6.10).

Differences in biomarker responses and specific (quantitative) histological changes in fish collected from different locations were examined using a one-way analysis of variance and Tukey's multiple comparison tests. Multi-dimensional scaling (MDS), an ordination technique that describes similarity (or dissimilarity) between pairs of units, was produced using a normalised Euclidean distance resemblance matrix in PRIMER 6 to show the gradient of effects extending from the contamination sources.

Results and analysis

Tetractenos glaber sampled in Sydney Harbour demonstrated higher biomarker stress responses than those from the reference locations. Fish from the highly contaminated Homebush Bay were under the greatest apparent physiological stress, based on biomarker responses. These fish demonstrated elevated EROD activity, reduced GST concentrations (Fig. 6.11) and signs of reproductive dysfunction (Fig. 6.12).

Biomarker responses in toadfish sampled from Five Dock Bay and Shell Cove indicated that these fish were also suffering from physiological stress. Higher EROD activity and reduced GST concentrations were characteristic of fish sampled from these sites (Fig. 6.11). Fish from Roseville Chase displayed elevated EROD activity and higher concentrations of lipid peroxidation (MDA).

Histopathological analysis of gonads in this study identified a range of differences in the appearance of toadfish testes collected from the different field locations and, importantly, some of these were degenerative changes that could be expected to negatively

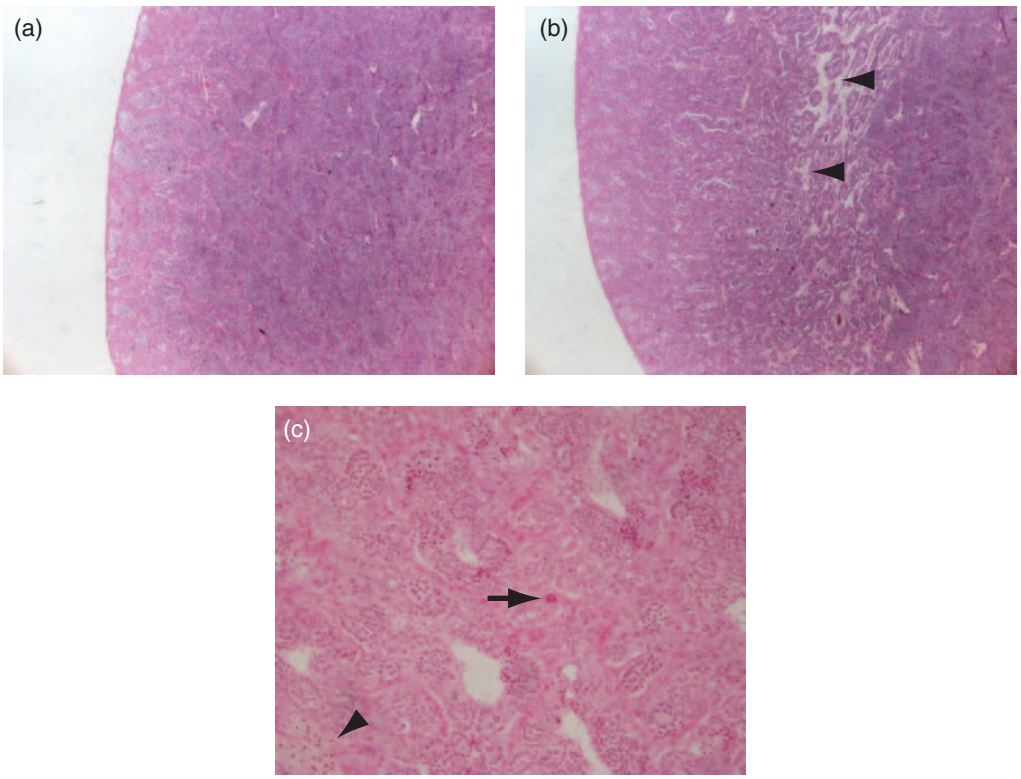

Figure 6.10. Male toadfish testis: (a) normal; (b) interstitial fibrosis affected (arrow head), magnification 100×; (c) germ cell syncytia (arrow) and pigmented cell accumulation (arrow head), magnification 400× (photographs: Dr Kathryn Hassell).

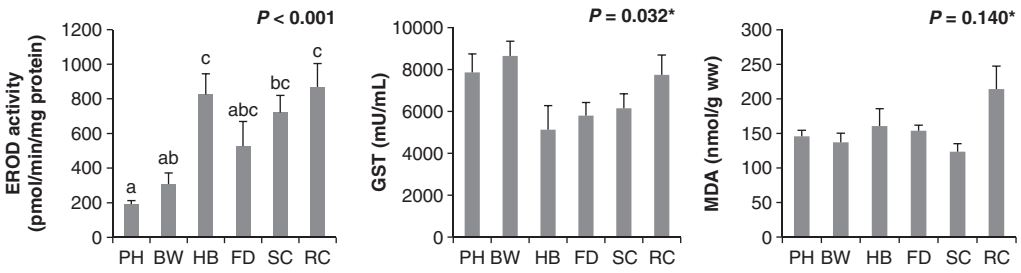

Figure 6.11. Biomarker responses: EROD activity (pmol/min/mg protein), GST (mU/mL) and MDA (nmol/g wet wt) concentrations (mean ± s.e.) in livers of male *T. glaber* sampled along an organic contamination gradient in Sydney Harbour (HB = Homebush Bay, FD = Five Dock Bay, SC = Shell Cove and RC = Roseville Chase) and at two reference locations (PH = Port Hacking, BW = Berowra Waters). Different letters denote significant differences ($P < 0.05$). * No pairwise differences observed.

influence reproductive output (fecundity). Male toadfish from Homebush Bay and Five Dock Bay had significantly higher numbers of pigmented cell accumulations compared to male toadfish from Berowra Waters and Shell Cove ($F_{(5,56)} = 4.7153$, $P = 0.0013$) (Fig. 6.12). There was a noticeable increase in the numbers of germ cell syncytia in some individuals from Homebush Bay (Fig. 6.12), but the difference was not significant in comparison to

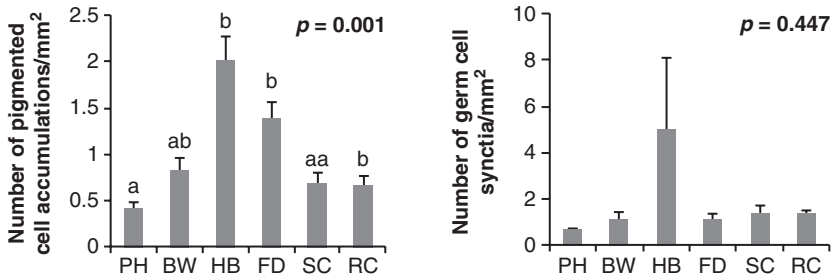

Figure 6.12. Number of pigmented cell accumulations and germ cell syncytia (mean ± s.e.) as a proportion of testes area (mm^2) in *T. glaber* sampled along an organic contamination gradient in Sydney Harbour and at two reference locations. Different letters denote significant differences ($P < 0.05$). * No pairwise differences observed.

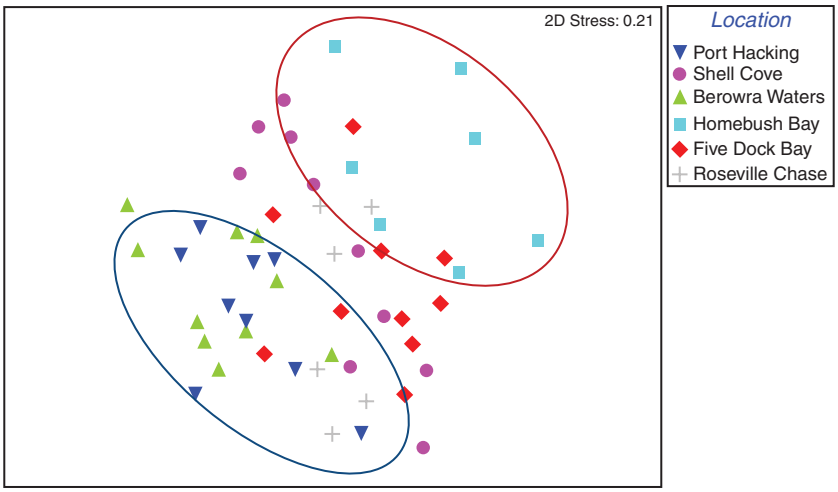

Figure 6.13. MDS plot of biomarker responses (EROD, GST, lipid peroxidation and gonad histopathology) measured in male *T. glaber* sampled along an organic contamination gradient in Sydney Harbour and at two reference locations. The red circle indicates the grouping of samples from the location with the highest contamination; the blue circle indicates the grouping of the samples from the reference locations.

other locations ($F_{(5,56)} = 0.9671$, $P = 0.4468$). The highest incidence and severity of interstitial fibrosis and vacuolisation of germ cells were observed in fish from Homebush Bay and Five Dock Bay, respectively.

The MDS (Fig. 6.13) indicates that a gradient of effects exists, extending from the highest organic contaminant source, Homebush Bay, to the reference locations, Berowra Waters and Port Hacking.

Quantitative biomarker indexes have been proposed to integrate the effects of biomarkers; see, for example, summary and application of existing indexes in Benedetti *et al.* (2012) and Schettino *et al.* (2012). These indexes provide alternative approaches for visualising and comparing between locations or surveying differences, providing a means of summarising complex biomarker data, resulting from multiple cellular pathways, into simple health indices that reflect the severity of damage or stress caused by contaminants. This provides scientifically sound, but also user-friendly, information for environmental managers and decision makers (Regoli *et al.*, 2014).

6.11 Concluding remarks: the future

In this chapter, an overview has been provided of validated biomarkers that are in wide use in risk assessment, together with a framework in which biomarkers can be used as a line of evidence, and factors that need to be considered when choosing organisms and designing sampling programs to measure biomarkers. Many new biomarkers, particularly genetic approaches including genomic, metabolomic and proteomic biomarkers, are currently being developed, and results outlining their potential application in toxicology have been published. Many of these biomarkers provide useful information to allow a deeper understanding of mechanisms linking dose and effects and, with further research, have the potential also to add value as lines of evidence in sediment quality assessment. Integration of the measurement of biomarkers into risk assessment programs is happening extensively worldwide because, when combined with contaminant analysis, biomarkers offer great diagnostic potential for assessing organism and ecosystem health.

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7

Ecological assessment

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7.1 Introduction: benthic bioassessment

An ecological perspective, where sediment contamination can be viewed against a background of more complex dynamic ecological patterns and processes, can aid in the interpretation of change phenomena in sediment-dwelling communities, in terms of inferring cause. This chapter provides guidance on the design, implementation, analysis and interpretation of studies examining the responses of communities of marine and freshwater benthic fauna to contaminated sediments. It gives a framework that can be adapted to a variety of scenarios, directing users to appropriate reference material that may assist in more specific matters or alternative approaches. Some of the more commonly-used study approaches (that is, reference condition approaches and environmental gradients), are critically reviewed and summarised, to demonstrate how to choose a relevant study design.

The chapter does not cover prospective (that is, pre-impact) study designs, where contamination is anticipated but has not yet occurred. Rather, attention is given to 'areas of concern', where contamination is already present and where the need is to determine whether ecological patterns and processes within natural communities in contaminated areas show evidence of adverse ecological change, and whether these ecological observations can provide a useful line of evidence or insight into the investigation of cause, as part of a multiple lines of evidence approach (see Chapter 1.4).

It is important to make a distinction between bioassessment approaches that provide general evidence of impact, and those that provide more detailed causal insights (Baird and Hajibabaei, 2012; Coffey *et al*., 2014). In field investigations, it is essential to keep in mind that, even in heavily-contaminated areas, a range of stress factors will inevitably be influencing any patterns observed within natural communities. In any study focusing on an unknown contaminant it is important to consider alternative hypotheses before drawing inference on matters of causality.

To illustrate bioassessment, the chapter gives examples of benthic studies from both freshwater and estuarine environments. The aim is not to be exhaustive, however, because this is not a review; for a review, see Burton and Johnston (2010). The intention here is to outline how to approach study questions rigorously, generating data that are compatible with a variety of statistical techniques, based on sound principles of experimental design.

Interpreting patterns in biological assemblages and communities from field studies is a task fraught with difficulty. This is particularly true where taxonomic knowledge is poor,

and identification of specimens is difficult. A partial solution to that challenge is the technique of DNA-based identification of organisms from bulk environmental samples (Chariton *et al*., 2010a, 2015; Hajibabaei *et al*., 2011; Yoccoz, 2012) – a significant new development that has emerged since the previous edition of this book was published. While this technique is still being refined, it has reached a stage where it can be applied in parallel with more traditional methods of identification. This chapter therefore includes advice on how to collect samples which can be analysed now or archived for future analysis by DNA-based identification and other newer approaches.

7.2 Benthic bioassessment in an ecotoxicological context

Benthic communities are a well-defined and extensively studied component of marine and freshwater ecosystems. They are critical sources of primary and secondary productivity, interact with and alter the physical and chemical condition of the sediment and sediment–water interface, and transfer biomass to higher trophic levels (Rowe, 1971; Gaston *et al*., 1998). Benthic communities inhabit and interact with sediment-associated contaminants through a range of exposure routes (Chapter 1 Fig. 1.2), are generally less mobile than pelagic fauna (such as fish), and can be sampled quantitatively. They exhibit a range of ecological characteristics and traits (White, 1988; Dauer, 1993; Warwick, 1993), and their direct and indirect responses to contaminants can include changes in the composition and relative abundances of taxa because of differential sensitivity (for example, Leung *et al*., 2005; Dafforn *et al*., 2013; Hill *et al*., 2013). Indirect effects may arise from altered recruitment, shifts in food web structure, and the cascading effects caused by the loss or increase of specific taxa (Attrill and Depledge, 1997; Fleeger *et al*., 2003). Their importance as the base of nearshore food webs and their sensitivity to toxic substances make studying benthic communities a critical component of sediment quality assessment (Bilyard, 1987).

Toxicity data gleaned from well-conducted standardised laboratory toxicity tests are generally more transferable, accurate and precise than field experiments. Yet, although such tests can provide relevant information on the toxicity of substances to sediment-dwelling organisms (see Chapter 4), extrapolating these findings for the protection of field communities is fraught with ambiguity (for example, Schlekat *et al*., 2005). This is a significant issue in most countries, because relevant toxicological data for local freshwater and marine taxa are few.

By contrast, community-level field studies are location-specific, objective-specific, subject to a high degree of spatial and temporal variability, and generally confounded by the issue of multiple stressors. Moreover, field studies are inherently expensive and time-consuming; thus, clear and unambiguous guidelines for their execution are essential. Poor study design or inappropriate methodologies inevitably lead to irrelevant, ambiguous and/or incorrect findings, failing to support sound management decision-making. Nevertheless, field studies provide the critical bridge to reality in sediment quality assessment, identifying the collective responses of a broad suite of taxa under ecologically relevant conditions.

7.2.1 Asking the right questions

Before developing an experimental design for a field study, clear and relevant hypotheses need to be established. For that, it is essential that the following questions are posed (for more details see Box 7.1).

(i) Why are you carrying out the study?
Clear guidance on the purpose of any investigation is essential to support the framing of clear study objectives, ideally as scientific hypotheses.

(ii) Do you have an understanding of the basic physico-chemical characteristics of the ecosystem being surveyed, in terms of relevant system properties and drivers?
This could include type, source, concentrations and movement (fate) of contaminants; hydrodynamics; sediment type; and geomorphology.

(iii) Do you have access to current knowledge regarding the ecological characteristics of the system being studied?
This could include taxonomic lists, knowledge of ecosystem function (for example, productivity and food-web connectivity).

If data are lacking to support (ii) and (iii), it will be necessary to carry out a baseline study to aid in selecting appropriate methodologies, and to frame relevant hypotheses.

Although they require effort and are costly, baseline studies can help to eliminate the risk of making comparisons with unsuitable reference locations, while ensuring that the ecological and environmental information obtained is at a prescribed level of confidence and relevant to the overall objectives of the study. Baseline studies are often ignored, being viewed as an unnecessary cost; yet the information they provide can help avoid costly mistakes.

The underlying question of a study may be based on identifying if there are differences in benthic assemblages between a contaminated location and several reference locations, but more specific hypotheses also need to be defined for use in testing the overall objectives. For example, it may be necessary to separate out other major factors in assemblage variation (such as sediment grain size), to ensure that any inferences drawn can be reasonably attributed to the stressor driving the study question. Moreover, hypotheses need to be testable under a statistical framework and contain an acceptable level of uncertainty (error).

Box 7.1: Asking the right questions (digging a little deeper)

What is the purpose of the assessment?
Place the study within a regional context. Identify any site-specific issues and minimum local data requirements. Is a baseline study required, to overcome a lack of local site data?

What is the nature of the sediment contamination?
What local or regional industries are current or past emitters? Are there inputs from point sources or diffuse sources? Is there evidence of guideline exceedances for specific substances at any of the study sites? Are documented exceedances still occurring, or were they observed in historical studies?

What other major habitat and stress factors could be influencing assemblage structure at sites?
Consider the salinity, temperature, pH, nutrients, grain size, contaminants which are not the focus of the study, and differences in other key habitat elements (such as low productivity, limiting food availability).

Are the physico-chemical data compatible with the biological data?
Were data collected at the same time, and at the same location? Are the driver data sufficient to describe potential influences on the biota? (For example, if there is only 1 year of driver data, is this enough to describe antecedent conditions underlying present biota patterns?)

7.3 Fundamentals of sampling and experimental design

The experimental design provides the framework for the study, establishing boundaries for trade-offs between the level of uncertainty and the costs and time committed to the project. By developing a well-designed study, the major variables that influence the level of uncertainty can be moderated, such as spatial and temporal variability, errors, and biases in statistical approaches. The analysis of spatial patterns is of primary interest for community ecologists examining the putative responses to contaminants and other stressors. As illustrated in this chapter, changes in the spatial patterns among sedentary biota are examined through field studies or surveys, also known as mensurative experiments (Hurlbert, 1984), with such approaches being founded on correlative relationships between the sampled communities and environmental variables. In contrast, experimental procedures, such as manipulative experiments (see Section 7.4 below), are required to examine cause–effect relationships (Chariton *et al*., 2011).

No prefabricated experimental designs are universally applicable for examining the relationships between benthos and environmental variables. Consequently, this section provides an overview of the basic concepts and principles that underpin the experimental design and sampling approaches for benthic surveys. In contrast to statistical tests which may be chosen to suit the specific attributes of the data subsequent to its collection, no such liberties are available with a poorly designed study – that is, one which is inappropriate for addressing the aims and hypotheses of the study.

Designing field studies and manipulative experiments is challenging and requires a balance between theoretical principles and the practicalities associated with costs, collection time and processing time (Kindt and Coe, 2005). Regardless of the final approach taken, it is critical that the sampling regime: (i) is sufficient to define the statistical population of interest; (ii) encompasses the correct spatial and temporal boundaries; and (iii) is founded on sampling units of appropriate type and size (Quinn and Keough, 2002).

To ensure the suitability of an experimental design the following recommendations should be carefully considered:

- the aims and hypotheses of the study are clearly articulated (see Section 7.2.1);
- consultation is sought with a suitably experienced professional biometrician before any design is begun;
- a pilot study is performed to provide preliminary information on the variation of both the biotic components of the system (benthic communities) and its abiotic components (such as contaminants, salinity gradient (for estuarine studies) and sediment type). This will enable refinements to be made to the area of the study, replication at various scales, and sampling units (volume of sediment and mesh size of the sieve). Such adjustments may not only maximise the utility of the data but also assist in delivering the best-value study.

7.3.1 Sampling

The underlying objective of a sampling program is to collect sufficient samples, and at temporal and spatial scales appropriate for the hypotheses being tested. Estuarine and riverine measurement studies are often performed over large areas that encompass a variety of different habitats (for example, areas of varying energy and substrate) and can experience marked variations in flow (for example, tides and seasonal rainfall events). Consequently, marked differences in the composition of benthic biota and the attributes of the measured environmental variables can occur across a range of spatial and temporal

scales (Morrisey *et al*., 1992a,b; Downes *et al*., 1993; Morrisey *et al*., 1994a,b; Vinson and Hawkins, 1998; Lammert and Allan, 1999). To accommodate this variability an appropriate experimental design and sampling regime are required. For field studies, the spatial scale is normally set using explicit biogeographical or morphological boundaries that are of ecological relevance (Kindt and Coe, 2005); for example, the main channel of an estuary or the fringing sediments surrounding a whole lake. It is important to recognise that the spatio-temporal limits of a study, also referred to as its extent, mean that any subsequent interpretations cannot extend beyond the actual area or time examined. For example, observations obtained exclusively around the fringe of a lake cannot be extrapolated to unsampled regions such as deep-water sediments in the lake's centre.

The selection of appropriate reference locations is a difficult task but nevertheless critical. In many studies, potentially-impacted locations have been compared to either a single location or a series of unsuitable locations: for example, reference locations that are still influenced by the contaminants (that is, are not independent), or that are historically contaminated, or that are subject to other disturbances such as stormwater inputs, eutrophication or large fluctuations in tidal salinity regime. Consequently, the findings of such studies may indicate that there is no difference between the contaminated and reference locations, resulting in the conclusion that the putative contaminants pose no significant ecological threat. This interpretation would be statistically confounded and possibly erroneous.

The null hypothesis (H_0) of an experiment is generally (but not always) that there is no relationship (or difference) between population parameters (Quinn and Keough, 2002); the parameters might be the population means of an unaffected location (no impacts) and an affected location (having impacts). Statistical testing of the null hypothesis can produce four outcomes: the correct acceptance or rejection of the null hypothesis, or the incorrect acceptance or rejection of the null hypothesis (Table 7.1). When H_0 is erroneously rejected (that is, a significant difference is detected when none is present), the error is referred to as a Type I error. When H_0 is accepted even though a significant difference exists, the error is called a Type II error. It is optimal to keep both errors as small as possible, but practical limitations such as resources require a compromise. Simply reducing the likelihood of a Type I error by increasing the α value (the value which determines at what level the probability (P) will be considered significant) will increase the likelihood of a Type II error. Generally, α is set at a specific level, such as $\alpha = 0.05$; this means that when $P \leq 0.05$, the null hypothesis is rejected.

From a risk-assessment perspective, it is the Type II error that requires careful consideration, because this may create the erroneous assumption that no impact has occurred, and therefore no remedial action is required (Quinn and Keough, 2002). Type II errors are influenced by variability, and hence are study-specific. This type of error can be reduced

Table 7.1. Statistical conditions and the types of statistical errors formed from null hypothesis testing

	Reject null hypothesis	**Accept null hypothesis**
Impact consideration:	Correct decision	Type II error
	Impact detected	*Impact not detected*
No impact consideration:	Type I error	Correct decision
	Impact detected, but none exists	*No impact detected as none exists*

by using appropriate experimental design, with sufficient statistical power to detect an effect (impact).

Statistical power is a complex subject, and the appropriate number of samples required to detect impacts of a particular effect size cannot be prescribed here because it is related to the magnitude of the effect that is occurring. This chapter gives a brief overview of sampling and considerations for designing experiments, but to minimise erroneous conclusions readers are referred to Quinn and Keough (2002) and Mapstone (1995) when planning studies.

As the distribution of macrobenthos is often patchy, increasing the number of replicates (discussed below) and/or the volume of sample may be required to maximise representativeness. Pooling of multiple samples into a composite sample and then sub-sampling is not recommended: first, it is impractical to sufficiently homogenise sediment without damaging organisms, a requisite for obtaining a representative sub-sample; and second, potentially useful information regarding small-scale variation (sample level) will be lost (see also Chapter 5 Section 5.3.1).

There are four commonly used sampling approaches: systematic, random, stratified and clustered (Fig. 7.1), as discussed also in Chapter 2.

Systematic sampling

Systematic sampling (Fig. 7.1a) uses even spacing between samples after the first sampling point has been randomly selected. As indicated by Quinn and Keough (2002), the approach may be useful in some scenarios, such as in identifying the position along a gradient where a pronounced change in composition has occurred. However, systematic sampling is susceptible to biases because the sampled gradient may include unmeasured or unforeseen co-occurring gradients (for example, changes in grain size and electrical conductivity) (Kindt and Coe, 2005). More commonly, systematic sampling is applied to the time (temporal) component of a sampling program, for example monitoring a site on a monthly basis. Again, patterns associated with temporal systematic sampling may be confounded by other factors operating at different temporal scales, such as rainfall events.

Random sampling

Random sampling (Fig. 7.1b), as the name implies, is the process where samples are obtained from randomly generated horizontal and vertical positions within the study region. In reality, pure random sampling may result in some of the system's components being over- or under-represented. For example, in Fig. 7.1b, random sampling resulted in only a small proportion of the 30 samples being collected in the transitional waters. While additional sampling will ultimately even out such biases, this may require an extensive number of samples and the production of a many data that are redundant with respect to the objectives of the study (Kindt and Coe, 2005).

Stratified sampling

Stratified sampling is an approach that divides the sampling region into non-overlapping strata (for example, fresh, transitional and marine waters). By targeting each stratum, the likelihood of obtaining relevant information is increased and redundancy is reduced (Fig. 7.1c). The approach is particularly useful when the stratum level is incorporated into the hypotheses, for example a hypothesis that species richness changes along a salinity gradient. The primary disadvantage of stratified sampling is that the boundaries of the strata must be known before sampling. As is evident in the case of a salinity gradient (Fig. 7.1), defining pragmatic boundaries to such strata may be difficult in an estuary for

example, because salinity concentrations will continually vary and the boundaries of the strata will change with tide and season. Sampling within strata can be performed in many ways, including using random or systematic sampling.

Cluster sampling

Another approach is to use cluster sampling (Fig. 7.1d), which aims to sample relatively homologous and naturally organised units within a hierarchy. In the example shown in Fig. 7.1d, three clusters each containing five pseudo-replicated samples (see below) are obtained within each stratum. The position of the clusters can be random within the stratum

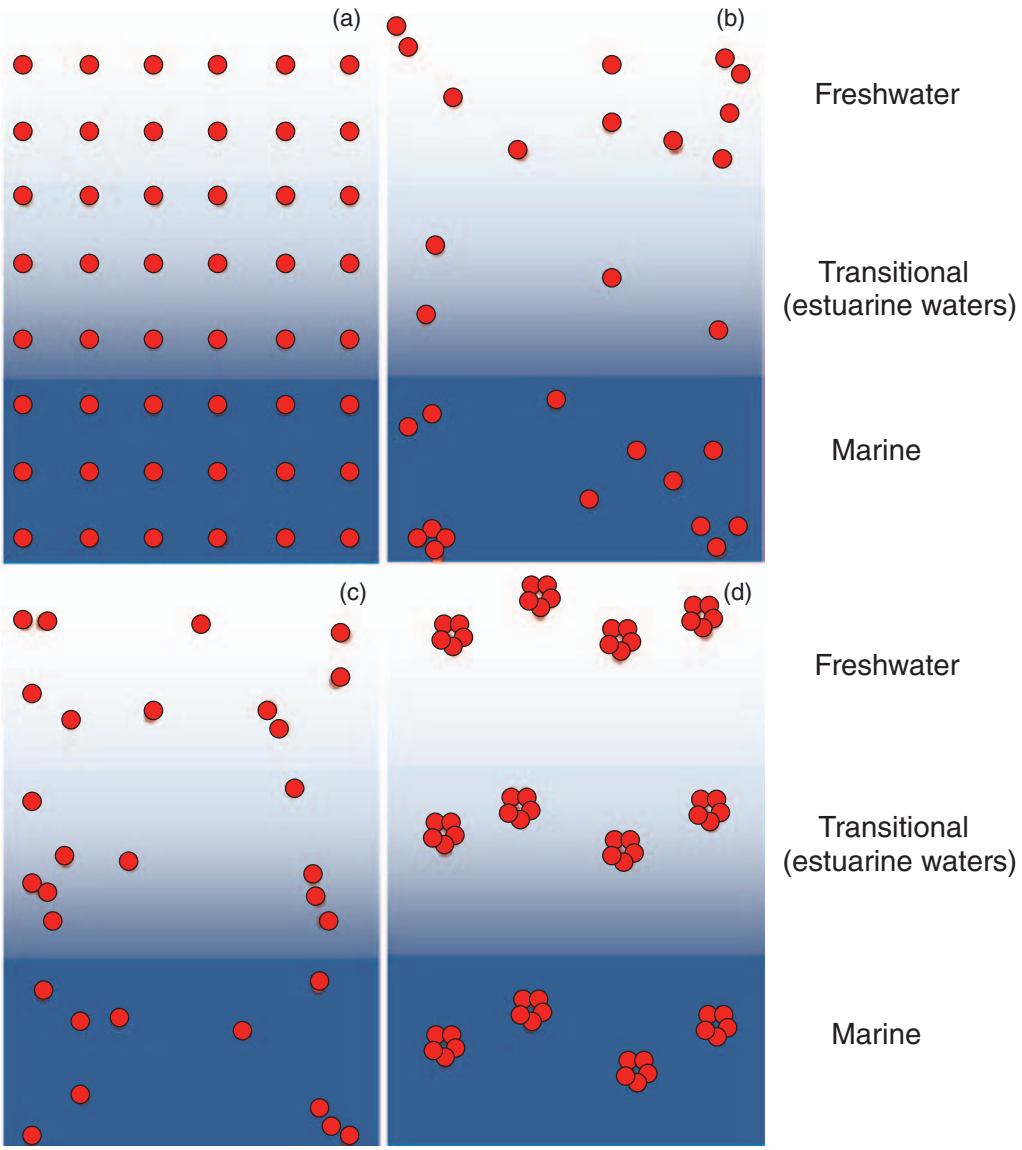

Figure 7.1. Four different approaches to sampling a system with distinct marine, transitional and freshwater environments: (a) systematic; (b) random; (c) stratified-random and (d) cluster.

(Quinn and Keough, 2002), but many studies space them equidistantly within a stratum (for example, Morrisey *et al.*, 1992a; Chariton *et al.*, 2010b) to ensure that the distance between clusters (such as within a bay) is less than the distance between strata (such as between bays). Cluster sampling is frequently used in estuarine studies as it forms the basis of nested designs (ANOVAs and PERMANOVAs) (see Sections 7.7.1 and 7.7.2 below).

7.3.2 Reducing confounding

Probably the most important consideration in a sampling program is the problem of confounding. In its most rudimentary form, a confounded design results in inability to separate differences in the compared sites (for example, impact versus reference sites) because of overriding influence from an additional factor(s). In estuarine systems, for example, a common confounding factor is sediment grain size, because the proportion of fines (<63 μm) is generally far higher in anthropogenically modified systems than in those that are predominately undisturbed (Chariton *et al.*, 2010b). Even without the presence of a contaminant, marked differences in grain size can have a pronounced effect on biological composition (Snelgrove and Butman, 1994). Therefore it may be difficult to confidently attribute any perceived changes in benthos to the contaminant, when comparing contaminated sites with clay or fine substrates to reference sites with coarse sands.

Even in clearly stratified regions, most riverine, lake and estuarine environments will contain a diverse array of habitats, with small-scale abiotic and biotic processes contributing significantly to the biological variability of the sampled location. Undoubtedly, field studies always contain co-variables which have the capacity to cause deviations within the main trends being examined, but their influence can be reduced by defining some limits on the key variables – for example, water depth, sediment particle size, seagrass cover, salinity and the concentrations of dominant physico-chemical properties of the water column. However, it is critical to ensure that the defined regions and conditions are present in all sites being examined and, more importantly, that the selected regions are the ones where the expected phenomena are most likely to be observed; for example, a change in benthic composition due to high concentrations of metals. While some statistical tools are available that enable practitioners to remove or deliberately include co-variables (see Section 7.7.4 below), it is important to understand the fundamental attributes of experimental design because no statistical tools can resolve a study that is truly confounded.

Ecological systems are variable, and multiple observations (samples) are required to capture the statistical attributes of a system – a process referred to as replication. As a majority of statistical techniques, including all of those described in this chapter, are founded on variation, the appropriate use of replication is pivotal. Importantly, replication can help eliminate confounding effects. For example, if a researcher simply compared one oil-impacted location versus one reference location, no firm judgment about the differences between the locations would be plausible because one or both locations could be confounded by another unmeasured factor. Sampling several oil-impacted and reference locations would provide a more realistic view of any differences. However, replication is not always as simple and tangible as the above example.

One of the most common issues in experimental designs is pseudoreplication. Pseudoreplication is defined as 'the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or experimental units are not statistically independent' (Hurlbert, 1984); or more succinctly, when statistical inferences are made on samples that are incorrectly assumed to be independent. The issue of pseudoreplication can manifest itself at all levels of design, but it

commonly occurs at the lowest level of replication. For example, in sediment sampling, when five cores are taken from a 1 m^2 area it is often incorrectly assumed that each of the five cores is an independent replicate, when in fact five psuedoreplicated observations have been made of a single replicate. A more detailed discussion of pseudoreplication and solutions for dealing with it are provided by Millar and Anderson (2004).

7.3.3 Reference locations and conditions

In survey studies, true controls (that is, locations that are identical to the impacted location with the exception of the contaminants) are unattainable. A more realistic notion is to accept that the system is modified, and choose multiple reference locations (that is, locations which are considered to be relatively unmodified or of similar high biotic integrity). Reference locations are chosen on the basis of a set of explicitly-defined criteria that will minimise the influence of confounding factors such as large differences in sediment particle size (grain size), nutrients, salinity, physico-chemistry, hydrodynamic processes, depth, and so on (Whittier *et al*., 2007). If comparisons are made between only two locations, for example near a smelter (contaminant source) and 10 km away in an adjacent bay (reference location), then any differences may not be linked to the smelter, and the assemblages may just be different. The likelihood that natural disturbances will obscure anthropogenically-induced changes in benthic assemblages is common in estuarine studies (see, for example, Wu, 1982; Nipper *et al*., 1998; Saiz-Salinas and Gonzalez-Oreja, 2000; Mistri *et al*., 2001; Morrisey *et al*., 2003).

In a comprehensive assessment of North Carolina (USA) estuaries, Hyland *et al*. (2000) found that 27% of the region studied contained impaired benthic assemblages although no significant concentrations of contaminants were identified, suggesting the influence of natural disturbances and/or unmeasured contaminants. These findings emphasise the need for multiple reference locations, and the difficulty in making assumptions about observed differences between contaminated and reference locations. For more detailed information on experimental designs for ecological studies, see Underwood (1994, 2000) and Quinn and Keough (2002).

An assessment of the condition of a river system, based on benthic macroinvertebrate data, will often involve conducting multiple surveys of sites from selected reaches. Often, the aim of these studies is to determine river condition by examining the fauna assemblage and determining what factors (such as catchment land uses, physical habitat, water quality and sediment quality) are influencing their community structure. The fauna assemblage at a site is usually compared to that at one or more reference sites, which could be in the upper catchment or neighbouring streams, depending on the purpose of the assessment. The types of macroinvertebrates present in a stream are known to change along a river from its headwaters to its estuary, as illustrated in the River Continuum Concept (Vannote *et al*., 1980), and therefore it would not be expected that a lowland stream would have the same fauna as those in a headwater stream or that either would be a good reference for the other.

In riverine surveys, multivariate predictive models have been developed to provide a reference condition. These models use macroinvertebrate data to measure impairment of stream condition. They were first developed in the UK (Wright *et al*., 1993) and have since been developed in North America (for example, Reynoldson *et al*., 1997; Hawkins *et al*., 2000) and Australia (for example Parsons and Norris, 1996). The AUStralian RIVer Assessment System (AUSRIVAS) has predictive models for all states and territories (Davies, 2000; Simpson *et al*., 2000). Hundreds of reference sites are used for developing these models. For instance, the New South Wales (NSW) model uses 200–300 reference sites (Hose *et al*., 2004). An AUSRIVAS reference site is selected based on predetermined criteria (see Davis, 1994)

establishing that the site has had minimal disturbance or is the 'best available' in an otherwise more severely disturbed landscape (Metzeling, 2001).

AUSRIVAS uses reference data from benthic or littoral habitats and from either a single season (such as autumn or spring) or from those seasons combined (Simpson *et al.*, 1997). The model compiles a site-specific list of predicted taxa based on the environmental data. Predicted taxa are those that have ≥50% probability of occurrence (Simpson *et al.*, 2000). The probabilities of these taxa occurring are summed to provide the number of expected taxa.

7.3.4 Common designs in benthic surveys

Nested or hierarchical designs

Nested (or hierarchical) designs incorporate randomly-stratified sampling, and are intended to encapsulate the variability at a series of scales. A schematic example is provided in Fig. 7.2, where each location is divided into four sites from which four replicates are taken, with the entire sampling regime being repeated on two occasions (times 1 and 2). Consequently, each location reflects the variation that occurs among the sites and within sites (4 replicates). Underwood (1997) and Quinn and Keough (2002) provide excellent reviews of nested designs and their corresponding statistical analyses. Nested designs are adaptable to a range of benthic studies, including examining differences between impacted and reference locations, and gradient studies where changes in benthic communities are examined along a gradation in contaminants.

Temporal changes are also important and need to be treated appropriately, but an experimental design that included both a spatial and a temporal nested sampling design would be exceedingly expensive and time-consuming. An alternative approach is to randomly select times across the duration of the study, as illustrated in Fig. 7.2. In most

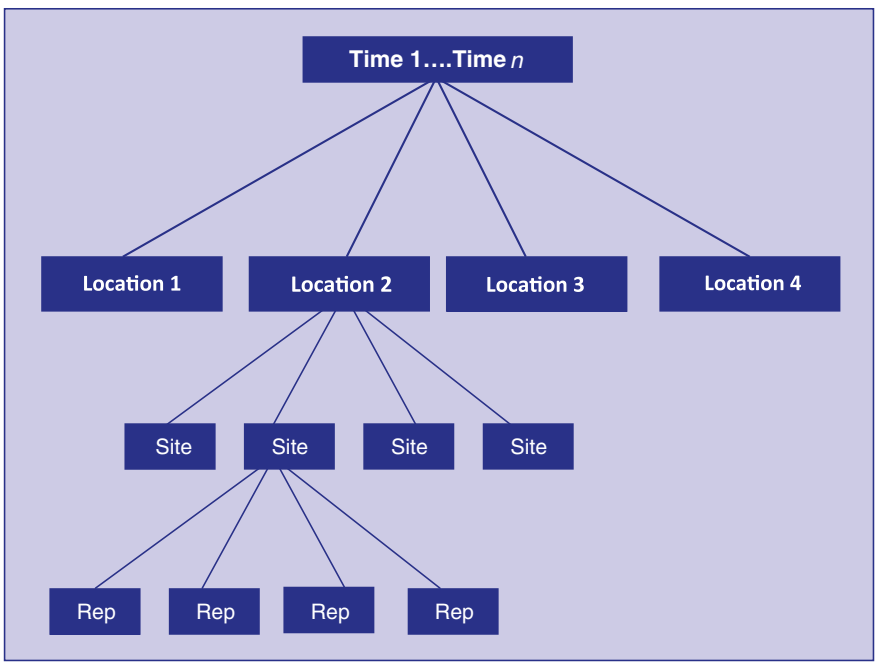

Figure 7.2. A nested design that is randomly replicated on *n* sampling occasions. Locations are fixed, with sites randomly nested within the locations and times, and replicates ('Rep') within the sites.

instances this would involve a stratified random approach. For example, if the study was only performed over a one-year period, randomly selecting the sampling dates across the seasons would provide a more representative sample of the variation than repeatedly sampling in one or two adjacent seasons. However, this would not show up a difference between seasons. In order for seasonal differences to be demonstrated, replication is required within and among seasons. For example, if the response of benthic communities was thought to be higher in summer than winter, then sampling should be randomly replicated within each summer and winter, and performed over multiple years.

Factorial designs

Factorial designs, such as the nested designed described above, are the most commonly applied approaches for measurement studies, as previously indicated, but there are some significant limitations associated with this approach. Most notably, factorial designs rely on the assumption that sites are categorically explicit; for example, contaminated and reference, or sandy and silty. In reality, there is often a gradient of contaminant concentrations, with continuous rather than categorical changes (for example, reference, moderately contaminated, highly contaminated). Furthermore, multiple gradients may be operating simultaneously, further disrupting any pre-conceived notion of formal categories.

Gradient studies

In contrast to factorial approaches, which ultimately struggle to reduce variability, an alternative approach is to employ an experimental design founded on detecting variability (Hewitt *et al*., 2007). For example, in a study exploring the relationships between macrobenthos, contaminants and environmental variables in Sydney Harbour, NSW, Chariton *et al*. (2010b) were able to clarify how various gradients in metals, organic contaminants and grain size correlated with macrobenthic community structure. As the locations contained a wide range of contaminants of varying concentrations, and had moderate differences in grain size and organic carbon, there was no simple or robust approach for categorising the locations. The approach taken not only provided a clearer understanding of the correlative patterns underpinning this complex environment, but also produced information showing which of the variables were pivotal or redundant, which was useful knowledge for subsequent monitoring of the system. Similar approaches have been applied to a number of macrobenthic surveys, including studies examining changes in macrobenthic communities in response to natural variables (Oug, 1998) and to contaminants along a salinity gradient (Peeters *et al*., 2000). As the approach explores the relationships between a suite of environmental variables and benthic community structure, it is crucial that sufficient environmental variables are measured concurrently, although the costs associated with obtaining a large number of environmental measurements can be prohibitive. Some of the key statistical techniques that underpin gradient studies are described in Section 7.7.4.

7.4 Manipulative experiments

Natural variability, sampling error, environmental covariates and additional (often unidentified) contaminants can affect the findings of survey studies, and reduce ability to confidently identify and assess the ecological risks associated with a particular location (Suter and Barnthouse, 1993; Batley *et al*., 2002). The information obtained from field surveys will always be correlative, and therefore cannot be used to determine causality unequivocally (Luoma, 1996). This remains true even when a single contaminant has been identified as the primary correlative stressor.

In practice, when comparing communities from reference locations and those with impacts, it is impossible to conclusively determine whether observed differences in benthic composition arise because of quantifiable differences in contaminants, or simply reflect the fact that the communities have been sampled from different locations. To clarify correlative studies and to increase our understanding of how contaminants affect benthic communities, the testing of models designed to elucidate cause and effect is essential.

One underutilised approach for obtaining an additional line of ecological evidence to tease out location-specific relationships between biota and contaminants is manipulative field experiments – specifically, translocation/recolonisation experiments. The underlying approach of these types of experiments involves the collection of sediments from multiple locations, with the sediments being translocated to a common site(s) of reference condition. In essence, these types of experiments use sediments collected from multiple locations and transported to a common location in reference condition for the study. Before translocation, the sediments are defaunated to remove all living macrofauna, generally by freezing or anoxia (Chariton *et al*., 2011; O'Brien and Keough, 2013). The defaunated translocated test sediments are then placed in containers, embedded into the sediment at the reference location, and left for a sufficient period of time to enable endemic biota to recolonise them. The retrieval step involves the macrofauna being collected from the sediments, enumerated and analysed. Statistical procedures for these experiments are similar to those applied to field surveys.

The underpinning premise of this approach is that the sediment, and not location, is driving observed differences in the composition and attributes of the recolonising biota between sediment types. Conversely, if no differences are detected between the biotic assemblages, it suggests that location-specific differences are overriding the changes observed in the associated field survey. While most recolonisation experiments have been performed in estuarine sediments, the approach is also applicable to streams and lakes (Pettigrove and Hoffmann, 2005).

The information obtained from such experiments can undoubtedly assist in partitioning out the confounding influence of location, but it is important to remember that any observed changes may still not be solely due to quantifiable differences in contaminants. Other attributes associated with the sediment (for example organic carbon content, grain size, ammonia and undetected contaminants) may also be contributing these patterns (Chariton *et al*., 2011). Using prepared sediments spiked with contaminants of interest (see Chapter 2 and Appendix C) in recolonisation experiments can overcome the confounding influence of varying sediment properties (Anson *et al*., 2008; Hill *et al*., 2011, 2013; Gardham *et al*., 2014a,b, 2015). These types of manipulative field experiment also provide insight into how exposure conditions change through time, because in energetic environments transplanted sediments often lose considerable amounts of material through resuspension, and they are buried by depositing suspended particulate matter in depositional environments (Hill *et al*., 2011).

It is also important to note that direct comparisons between the biota obtained from the field survey and the recolonisation experiment must be tentative, because the primary taxa identified in field surveys are from established communities which generally differ from those observed during the early stages of recolonisation, as typified in the manipulated treatments. Pronounced differences between the field-surveyed and recolonising fauna may also be due to artefacts associated with the containers, and the season and duration of the experiment (Hayward *et al*., 2001; Chariton *et al*., 2011).

An alternative approach instead of placing manipulated sediments into the field is to relocate field sediments, including their established communities, to the laboratory and

perform manipulative experiments in microcosms. There are variations on this approach, and one interesting and practical method was that developed by Chandler *et al.* (1997), where a layer of contaminated sediment was placed over the established communities and survivorship was measured by examining those organisms that successfully migrated to the oxic surficial sediments after a period of time. More recently this approach was modified by Ho *et al.* (2013) and Chariton *et al.* (2014) to examine the effects of the antibacterial agent triclosan. These studies obtained their data by using traditional optical techniques (meio- and macrofauna) and metabarcoding, respectively.

7.5 Collection of samples

7.5.1 Obtaining a suitable sample

Benthic communities can be sampled using both corers and grab samplers. The choice of device is dependent upon the environment being sampled, field conditions, and the methods used in other comparable studies. With both devices, effort should be made to ensure that the same portion of the sediment column is sampled and consistent volumes of sample are collected; that is, each sample must penetrate to the same sediment depth and contain the same volume of sediment.

Standardisation methods (for example, individuals/m^2) can be employed to correct for differences in sample volumes, but such corrections have some fundamental limitations. For example, increasing the volume of sediment not only increases the number of individuals that are sampled but also the likelihood of sampling rarer fauna, and thus influences the number of taxa sampled. Standardising sample volumes only corrects for differences in abundances and not for the number of taxa. While rarefaction – the recalculation of richness to accommodate variation in abundances among samples – is sometimes provided as a measure to correct for sampling effort, serious consideration is required to decide how this may affect the statistical outcomes of the study (McMurdie and Holmes, 2014). In addition, benthos may not be evenly distributed throughout the sediment column, with significant differences occurring along the sediment's vertical and horizontal profiles. Consequently, a simple correction for volume may not encapsulate stratified differences in the abundance and composition of the benthos.

Hand corers are suitable for inter-tidal areas and wading waters, and can also be used by divers in sub-tidal regions. Piston and gravity corers can also be used in sub-tidal regions. As previously indicated, the depth of the cores obtained must be consistent. The number of individuals and taxa that will be obtained from a core is highly variable, and ultimately dependent upon the site being sampled. In many instances, a core diameter of 100 mm will provide samples that cover a sufficient surface area, although this may not be the case in some depauperate environments. If the core diameter is too small, the number of taxa sampled may be reduced, especially in the case of larger or rarer species.

In silty or muddy sands, a core depth of ~100 mm is generally sufficient to obtain a representative sample. In all cases, it is preferable to perform a pilot study as a means of identifying the relationship between depth and assemblages, as well as identifying specific features of the site that may influence the depth of the cores, for example shell layers, and hard substratum. To assist in obtaining cores of a consistent depth, a plastic ring can be attached to the outside of the core tube to prevent it being pushed too far into the sediments (Fig. 7.3). Alternatively, clear Perspex® core tubes can be used, although these can be susceptible to shattering.

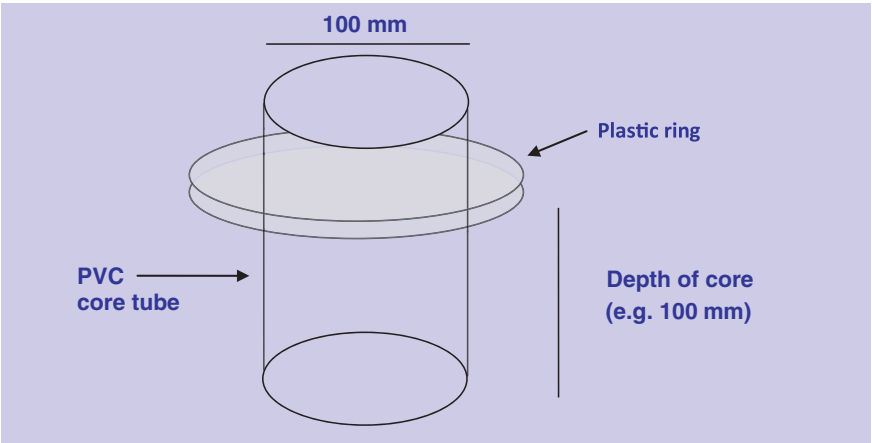

Figure 7.3. A 100 mm diameter PVC coring tube with a plastic ring attached to ensure that the core depth (100 mm in the example) is consistent between core samples.

Grab samplers (for example, van Veen and Eckman) can be used in sub-tidal environments. Depending on their size, a single grab can cover between 0.02 m^2 and 0.5 m^2 of sediment, and penetrate the sediment to a depth of up to 15 cm (USEPA, 2000). In most grab samplers the penetration depth is adjustable, increasing the precision of samples, especially under varying sedimentary conditions. Grab samplers enable a greater volume of sediment to be sampled, and consequently may be preferable to cores in environments where biota are in low abundance. However, many grab samplers are heavy, and therefore require an appropriate winching system. Furthermore, the volume and penetration depth may vary due to the penetration angle of the grab and differences among sediments.

To ensure that grabs are consistent and suitable for benthic sampling, the following criteria should be accounted for:

- sediment has not extruded from the sampler;
- water is still present in the sampler (that is, the grab remained closed during retrieval);
- the sediment surface is relatively flat; and
- appropriate sediment penetration has occurred: 4–5 cm in medium-coarse sand, 6–7 cm in fine sands, and >10 cm in silty sediments (PSEP, 1987).

USEPA (2000) provides an overview of grab samplers, including their suitability under various conditions, and the protocol for obtaining suitable samples (see also Chapter 2).

A range of sampling devices are available to collect macroinvertebrates in inland waters. The types of samplers used depend on the habitat being sampled and the type of data required. Riffles may be quantitatively sampled using a Surber sampler (for example, Marchant *et al.*, 1984) or a Hess sampler (for example, Pettigrove, 1989). A 'kick net' can be used to qualitatively sample the riffle fauna, but semi-quantitative data can be obtained if standard bioassessment sampling methods, such as AUSRIVAS, are employed. Wadeable pools and littoral zones can be quantitatively sampled using an Airlift sampler (Pettigrove, 1989) or by using artificial substrates that are deployed in the stream for 4–6 weeks (for example, the River Murray biological monitoring program in eastern Australia has used artificial substrate samplers as well as sweep nets; MDFRC, 2015a). Qualitative pool and littoral zone samples can be collected using a sweep or dip net. Again, as in riffles,

semi-quantitative samples can be collected using such a net if standardised methods are followed. For further details on these, see EPAV (1998) and the sampling manuals available from the AUSRIVAS website (AUSRIVAS, 2015). Deeper slow-flowing waters in rivers and lakes are usually quantitatively sampled using a Birge-Ekman grab. The surface of large woody debris can be sampled using a Snag Bag (Growns *et al*., 1999).

For sampling in coastal waters (2–20 m depth), grab samplers can be too heavy to transport to remote locations or to use on small vessels (for example, punts). In addition, these types of devices are not ideal for obtaining DNA-based data, because using the same device for all samples can lead to cross-contamination. Even though measures can be taken to reduce cross-contamination, for example only obtaining the sample from the middle of the grab, or using a butane torch to burn the surface of the grab between samples, it is more desirable to have a device that is interchangeable with every sample. Devices such as the Uwitec corer (UWiTEC, 2015) are ideal for coastal work, especially when using DNA-based approaches. These corers are relatively light, use PVC tubes that can be rapidly interchanged for each sample, and enable sediment cores to be easily obtained under a wide range of conditions, including sandy sediments and fast moving overlying waters. To minimise contamination, all coring devices should be pre-soaked in 10% bleach for 2–3 h, thoroughly rinsed five times with deionised water and individually wrapped using plastic food 'cling' wrap.

7.5.2 Fixing and preserving macrobenthic invertebrates

Once collected, the sediment should be removed from the sampler before fixing. Samples containing the benthos (benthic fauna) should be placed on a plastic mesh sieve (0.5, 0.75 or 1 mm) and rinsed gently to avoid damaging the animals. The mesh size should be the same as that of the sampling net (if used); (for example, for AUSRIVAS sampling the standard mesh size is 0.25 mm). Ideally, this step should be done in the field, to reduce the amount of sample and to minimise damage to the organisms during transportation. The retained fauna are then carefully transferred into a labelled air-tight container that is about twice the volume of the sample, and a 7% buffered formalin solution is added to fix the contents. (Note that some laboratory facilities preclude the use of formalin in the collected samples.) For marine and estuarine biota, the solution should be made up in seawater. For freshwater samples, deionised water should be used. No more than half of the container should be filled by the sample, and the rest of the container is then filled to the top with the fixative, to minimise sloshing and therefore damage during transport. The containers should be carefully inverted several times to enable the fixative to penetrate the whole sample.

The addition of a vital stain to the formalin solution (for example, 4 g/L of Rose Bengal) can dramatically aid sorting, though some taxonomists have found the stain to hinder identification; however, this is not generally a problem at family level. Before sampling, the decision to use or not use a stain should be discussed with the persons who will perform the identification.

Each container should be labelled, ideally with a waterproof tag inside the container with the sample. Formalin and alcohol (used for preserving) will erase 'permanent' marker, so details of the sample should be written on the tag with pencil instead.

Concentrated formalin can be buffered by adding 28 g/L of borax (sodium tetraborate); this solution has a shelf life of approximately 3 months. Alternative buffers such as sodium bicarbonate (40 g/L) can also be used, although these may leave a precipitate on the fixed animals. Formalin is a carcinogen, and therefore it is essential that it is poured in a well-ventilated area, preferably in the field, and that the handler wears gloves. In the field, samples

must be kept in a cool, dark environment. After a minimum of two days, the formalin-preserved samples should be placed on a fine mesh (such as a nylon stocking) in a fume hood and rinsed several times with a 70% ethanol solution (preservative) to replace the formalin. As formalin's acidity increases with storage time, samples should be transferred to the ethanol solution within 10 days of collection. If the samples are not sieved in the field (that is, the samples still contain sediment), compaction may occur within the containers, especially with soft fine sediments. In this case, the containers must be gently rotated every 48 h to ensure that the formalin has evenly penetrated the compacted sediment.

Ethanol-preserved samples should be sorted underneath a dissecting microscope to ensure that taxa that reside in sediment cases (that is, unstained casings) are enumerated. Only whole individuals are counted. All animals should be preserved in ethanol with the exception of Ophiuroidea (for example, brittle stars and basket stars). This class of taxa needs to be air-dried for identification (PSEP, 1987). A guide to sorting is beyond the scope of this book, although, Birkett and McIntyre (1971) and Beesley *et al.* (2000) provide useful overviews for the novice. See also Section 7.6.1 below for considerations of size and taxonomic resolution that should be decided before sorting.

In storing the samples, the fundamental priority for the preservation of DNA is to retard its breakdown. The approach used for storage will depend on whether the users wish to sort the samples from the matrix initially (for example, to live-sort specific taxonomic groups) or whether they wish to examine the bulk constituents of the samples (for example, the whole sediment sample). For samples sorted initially, the most common approach is to place the sorted specimens into 95% ethanol (Hajibabaei *et al.*, 2011), which enables users to identify the organisms before processing. Ethanol, however, must be removed before DNA extraction, and that removal process generally involves using a sodium acetate precipitation step (Dowton and Austin, 1994) which adds both time and cost to the procedure. Consequently, in cases where bulk environmental samples are collected for *en masse* sequencing, a more efficient approach is to freeze the samples immediately upon collection (at –80°C), thereby avoiding the effort required to remove the ethanol. Samples should be transferred into cryo-tubes and frozen immediately on dry ice or in liquid nitrogen using a dry-shipper (a spill-free container for transporting samples stored in liquid nitrogen). Samples should remain unthawed until they are ready for DNA extraction. Ultimately the samples should be stored as DNA extracts (at –80°C) and not as bulk sediments, because this not only saves on freezer space but also enables the samples to be stored and preserved indefinitely.

7.5.3 Quality assurance/Quality control

Correct identification of organisms

Where biomass is measured, weighing balances must be calibrated on a weekly basis, and microscopes must be regularly serviced. Staff will require initial training by taxonomic experts, and should have up-to-date taxonomic keys at their disposal. Staff should also participate in regional standardisation programs, if available (PSEP, 1987). Confirmation of taxonomic identification should be performed both internally and externally, with at least 5% of the samples re-identified by an additional qualified internal staff member, and at least three of each taxon being verified by an external benthic taxonomic specialist (PSEP, 1987). Externally verified taxa (reference taxa) should be kept in the laboratory to enable comparisons to be made throughout the identification processes, and for future reference through the creation of a reference library. The reference collection's integrity

must also be regularly monitored, with the vials containing the samples being regularly topped-up with ethanol to prevent desiccation. Full-strength ethanol (95%) is preferable under these conditions because of its relatively slow evaporation rate (PSEP, 1987). Filling the mouth of the individual vials with cotton wool will reduce buoyancy within the container, and prevent the loss of the vials' contents.

Each taxon from each sample should be placed in its own vial, and labelled appropriately. All specimens (including composites) should be labelled in such a way that the information is interpretable by any potential users. Basic information should include:

- the name of the taxon,
- date identified,
- survey project,
- site location,
- habitat the sample was collected in,
- sampling device/method,
- date collected,
- sorter's initials, and
- whether the taxon/taxa has/have been externally validated or not.

All vials for a given taxon should be subsequently stored in a single large jar containing 70% ethanol, and sealed to reduce evaporation.

Some studies have suggested that 20% of each sample should be re-sorted for QA/QC by an additional internal specialist (PSEP, 1987). For this step it is necessary to obtain a representative sub-sample (approximately 20% of the sample's volume) subsequent to it being spread on a sorting tray, and before it is sorted. The additional specialist should carefully re-examine the aliquot under a microscope, using magnification 25×, to ensure that an adequate percentage of individuals was initially sorted and correctly identified.

The level of accuracy required in taxa identification is generally determined by the facility, and is often dependent upon taxonomic resolution. The QA/QC for sorting and identification of freshwater samples will depend on the sampling protocol used; for example, for AUSRIVAS see Humphrey *et al*. (2000). In no cases should it be permissible for more than 5% of the taxa be incorrectly identified by the initial sorter. If >5% are incorrect, the sample should be re-sorted. This procedure, in concert with the internal and external confirmation procedures previously described, should result in very few, if any, taxonomic errors.

QA/QC in DNA-based assessments

Contamination is a primary concern for DNA-based assessments, because miniscule amounts of DNA invisible to the naked eye have the potential to reduce the utility of the data. It is therefore essential that stringent QA/QC procedures are implemented in both field and laboratory protocols. During field sampling, contamination can be monitored using 'field blanks' consisting of 'DNA-free' sediment created by autoclaving sediment or heating it in a muffle-furnace and storing it in clean vials. Attempts should be made to amplify the targeted products from the sample to ensure that it is 'DNA-free'. Field blanks should be treated as a normal sample in the field: that is, opened during sampling for the same period as is required to collect the sample. The field blank should then be transferred to the same container (for example, a dry shipper) as the other samples being transported. In the laboratory, the care used to monitor potential contamination should be similar to

that used in the field. During this phase, additional field blanks and water blanks containing DNA-free water should be used. Again, the samples should be processed in the same way as the other sediment samples; this includes DNA extraction and amplification.

Field blanks should be subjected to amplification processes, along with the test samples, to maximise the chance that DNA contamination will be detected. If the targeted products are found by amplification of the blanks, then these potential sources of contamination may be identified and, in appropriate cases, the contaminant sequences can be removed from the final analysis. In some cases, the sequences may not be a result of sampling or laboratory protocols, but rather reflect the materials used in the extraction and amplification kits: these potentially include sequences associated with pig, chicken, wheat and yeast (Chariton, unpublished; Pierre Taberlet, pers. comm.).

When an analytical facility provides results of chemical analyses, it is assumed that they will include information on the detection limits and the recoveries from reference materials. Without such information the data would be considered to be tenuous and unsuitable for environmental monitoring and assessment. However, to date, molecular ecologists have rarely employed such measures. To address this situation, it is advisable for several samples to be allocated to sequencing DNA from known taxa and at varying concentrations. These sequences could be generated either from artificial assemblages created from clones of known taxonomic origin (for example, Morgan *et al.*, 2013) or by using amplified tissue from species confirmed by a taxonomist (for example, Chariton *et al.*, 2014). The information derived from this sequencing can be used to identify where cut-offs should be made for the number of 'reads' (when serial dilutions of the reference samples are used), or to adjust the level of clustering (for example, 3% identity), and to refine the cut-offs for the taxonomic allocation of operational taxonomic units. As all bioinformatic pipelines have limitations, the only possible means of calibrating the tools and understanding the extent of the limitations is via the sequencing of material of known providence and concentration.

7.6 Taxonomic resolution of macrobenthic fauna

7.6.1 Taxonomic resolution when using traditionally collected macrobenthic data

Marine, estuarine and inland waters can contain a wide and often dynamic range of salinities that can have a pronounced effect on the abundance and types of taxa. Consequently, boundaries need to be established for the types of fauna that should be included in analyses. For example, in environments that maintain salinity close to that of marine waters (31–35‰), freshwater fauna could be excluded from the data matrix, if they are deemed unrepresentative of the sampled assemblages; similar exclusion could apply to pelagic taxa such as cladocerans and copepods. In marine or estuarine waters, the larval forms of benthic taxa are also generally excluded from the matrix because they are extremely difficult to identify; any changes in larval responses are aggregated into the overall attributes of the sampled communities. In systems with lower, or more dynamic, salinity ranges, location-specific boundaries need to be considered, in order to limit the taxa that should be sampled. This will require a local knowledge of the benthos, which can be developed either through previous studies or a pilot survey.

In many studies examining ecosystem contamination in marine and estuarine sediments, analyses at taxonomic levels coarser than the species level have resulted in a minimal

loss of information, often aiding discrimination by reducing the confounding effect of species replacement along environmental gradients (Warwick, 1988; Somerfield and Clarke, 1995). Identification and enumeration at family level or higher significantly reduces processing time and costs. Even in studies employing data with coarse taxonomic resolution, it is essential for staff to be properly trained in order to correctly identify taxa, with confirmation of the taxonomic identifications being validated by a professional benthic taxonomist. In specific cases, more detailed taxonomic identification may be essential, for example in cases where the status of a particular at-risk species is the focus of a study, or where complementary toxicity tests are being performed using taxa endemic to the study location. In these cases, professional taxonomic assistance is essential.

The taxonomic resolution used in freshwater macroinvertebrate studies is often influenced by the cost of identifying taxa and the need for specialist taxonomists. The early macroinvertebrate studies in Australia, such as those by Marchant *et al.* (1984) in the lower La Trobe River, Metzeling *et al.* (1984) in the upper La Trobe River and Pettigrove (1989) in the Yarra River, were quantitative studies that identified the fauna to the lowest taxonomic unit. Few animals could be identified to species level. Dipteran larvae were particularly problematic because the taxonomy was based on adults but the aquatic stages were usually larvae that had few morphological characteristics. The Elmidae were also a problematic group because the larvae and adults inhabited streams but few larvae and adults could be identified as one species. Although these studies yielded high quality data, they were expensive and only a small number of sites could be surveyed.

In the USA in the mid-1980s, the need for cost-effective biological survey techniques was realised because of the rapidly dwindling resources for monitoring and assessment and the need to assess large numbers of river systems. The first 'rapid bioassessment protocols' were developed by Plafkin *et al.* (1989) in the USA, and by Wright *et al.* (1984) in the UK, and in Australia during the 1990s (Parsons and Norris, 1996; Tiller and Metzeling, 2002). These rapid bioassessment protocols also tended to use family-level identification to reduce processing and identification of animals. Barbour *et al.* (1999) reasoned that identifying animals to a relatively coarse taxonomic level (such as family) provided more accurate information on ecological and environmental relationships and sensitivity to impairment, and that family-level identification provided a higher degree of precision among samples and taxonomists, required less expertise to perform and accelerated assessment results. In Australia, family-level identification was encouraged because family-level metrics were developed to aid data analysis. These include the biotic index SIGNAL (Chessman, 1995) and the use of number of families in compliance reporting (Victorian Government, 2003). Since the 1990s, there have been considerable advances in the taxonomy of many Australian macroinvertebrates and excellent keys plus training courses are available: see AUSRIVAS (2015) and the Murray-Darling Freshwater Research Centre's interactive 'bug guide' available online (MDFRC, 2015b). Family-level identification appears still to be the most common level of taxonomic identification used in freshwater surveys.

In addition to taxonomic resolution, the minimum size of the fauna to be collected must also be established. The size is defined by the mesh size used to retain the fauna before sorting; sizes commonly used are 250 µm (AUSRIVAS), 500 µm, 750 µm and 1 mm. In most applications the 250µm or 500 µm mesh may be the most suitable, potentially retaining more individuals and taxa than the coarser sieve sizes. However, decreasing the mesh size will increase the catch and therefore the processing time, and hence trade-offs between processing time, number of replicates and macroinvertebrate community representation need to be considered.

7.6.2 Taxonomic resolution in metabarcoding studies

For DNA-based (metabarcoding) surveys, the issue of taxonomic resolution is also important because this will dictate the choice of genes and loci, and then the need for a reference database. The 18S rRNA gene is generally used for studies which aim to capture a wide breadth of eukaryotic biota. However, the trade-off here is that taxonomic resolution can vary greatly (from genus to kingdom) and the quality of the taxonomic assignment is dependent upon the quality and availability of data within on-line genomic repositories (open-access sites which retain comprehensive sequence databases) such as GenBank (GenBank, 2015) and SILVA (SILVA, 2015). The options for species- or genera-specific information are more varied, with regions of the cytochrome c oxidase subunit 1 (CO1) gene often used to target specific taxonomic groups such as nematodes, Ephemeroptera and Trichoptera (Hajibabaei *et al*., 2011; Carew *et al*., 2013; Gibson *et al*., 2014). However, it is important for users to weigh-up and assess their choice of genes or loci, taking into consideration the availability of a reference database, taxonomically verified species, composition of the sample and the read length of the sequencing platform. Consequently, other genes may be more suitable than CO1, or provide greater taxonomic certainty and coverage when combined or used in tandem.

Several software packages are available to assist users in identifying and testing suitable primers from a range of genes, for example EcoPrimer and EcoPCR, which enable users to custom design and test primers *in silico* (Ficetola *et al*., 2010; Riaz *et al*., 2011). Approaches that require species-specific information will ultimately require a reference database, enabling specific variants of the targeted gene to be cross-referenced to taxonomically verified specimens. Ideally, for environmental monitoring, a composite approach is recommended, which includes genes with a broad taxonomic coverage (for example 18S rDNA) as well as genes that can provide more detailed information on key taxonomic groups of interest (for example, polychaetes and mayflies).

7.7 Identifying and measuring ecological stress

Interpretations of responses by macrobenthos communities to contaminant-induced stress (or perturbation) are derived from theories of ecological disturbance and general hypotheses associated with anthropogenic disturbances. Community responses may not be contaminant-specific. For appropriate correlative patterns to be identified between potential causes of disturbance and the communities, the relevant environmental (abiotic) measurements must be collected simultaneously with the biotic measurements. This collection must include natural variables (such as salinity, particle size distribution in the sediments, and dissolved oxygen) and contaminants. The high costs and time associated with collecting and analysing chemical data make it essential to have some prior knowledge of the potential contaminants, their concentrations and distributions through the sediments and waters.

Several approaches are frequently used to assist in discriminating between stressed and unstressed benthic communities. Two of these are discussed here: univariate measurements of communities and multivariate measurements. In addition, some approaches for linking benthic and environmental datasets are also presented.

7.7.1 Univariate measurements

Univariate measurements are frequently used to summarise the attributes of a community. They generally include the total number of individuals; indices for richness, diversity, and evenness; and the abundance of *a priori* selected taxa (for example, see Box 7.2).

Generally, these variables are relatively simple to calculate and can be analysed with several commonly-used statistical techniques (for example analysis of variance (ANOVA), and correlation and regression analyses). To provide statistically-robust interpretations, metrics need to be able to show a high level of response to contamination, and possess a low level of natural variability, thereby reducing the uncertainty associated with a small number of samples.

In some studies, the abundance of specific taxa is used as an indicator of stress. This response is often considered to be bi-directional; for example, an increase in opportunistic taxa such as the polychaetes Capitellidae (Grassle and Grassle, 1976; Pearson and Rosenberg, 1978; Tsutsumi, 1990), or a reduction in contaminant-sensitive taxa such as amphipods (Rand and Petrocelli, 1985; Warwick, 2001). It is important to select the indicator taxa *a priori*, before sampling, because *post-hoc* sifting through the collected data as a means of identifying patterns violates the concept of hypothesis testing, creating bias and often producing ecologically-irrelevant or confounded findings. Analyses based on the abundance of rare or spatially and temporally dynamic taxa should be treated with caution, because the estimate of abundance will be strongly influenced by many factors, such as recruitment, seasonal variation and sampling design, which will increase the level of uncertainty pertaining to the findings. Furthermore, data on rare or patchily-distributed taxa are likely to depart significantly from the assumptions of normality and homogeneity of variances which underlie standard parametric techniques of statistical analysis, making such techniques inappropriate.

The differential loss, replacement, proliferation and relative abundance of taxa are represented in indices of diversity, richness and evenness, and a decline in these indices is often symptomatic of ecological stress (Newman, 1998; Rogers and Hsu, 2001). The underpinning assumption is that contaminants can induce stress that affects the structure and stability of communities, which is subsequently observed as a change in the number of species and their relative abundances (Krassulya, 2001). The simplest measurement of diversity is species (or taxa) richness. However, this measurement has three serious limitations (Krebs, 1994): (i) it is improbable that all species will be counted; (ii) the boundary for the community being quantified is arbitrarily defined in space and time; and (iii) the measurement negates the concept of heterogeneity, and the relative abundance of each species.

Importantly, total taxonomic richness measured using DNA-based approaches (metabarcoding) also appears to be a poor indicator of environmental condition. Chariton *et al*. (2010a) in a study in Sydney Harbour found the largest increases in richness occurring at sites where multiple sources of biota are present; for example, the convergence of an estuary and a tributary. These sites often contain elevated concentrations of contaminants derived from catchment or upstream enterprises (such as sewage treatment plants). Consequently, the high increase in DNA-derived taxonomic richness at such sites is likely to be the result of a combination of two sources and an increase in degraded biological material which is still inadvertently amplified using the approach (Chariton *et al*., 2015).

Alternative measurements of diversity have been developed which individually measure or encompass the concepts of richness, sample size and evenness, producing an array of parametric and non-parametric indices (for example, the Shannon-Wiener Index, Simpson's Index, Pielou's Evenness). Numerous studies have shown a strong correlation between many of these variables and variations in the concentrations of contaminants, and consequently these measurements continue to form the basis of many benthic community studies (Johnston and Roberts, 2009). An example from a field survey along a

poly-metallic contamination gradient (Lake Macquarie, NSW, Australia) (Chariton, 2005) is illustrated in Box 7.2.

Such indices must be used with caution, and appropriately. As is the case with other measurements derived from sampling, the indices are influenced by the sampling method, habitat (for example, sediment type, geomorphology, vegetation), sample size, sampling depth, duration of sampling, time of year, and taxonomic level (Hughes, 1978; Washington, 1984). As a result, community index values can only be compared between locations sampled within the same study and under similar natural environmental conditions. In addition, sampled communities may have similar scores for, say, diversity, yet stark differences in taxa. In isolation, no single community index can be used to deduce a toxicological response at a

Box 7.2: Univariate measurements of the biota used in the analyses of benthic communities from Lake Macquarie (Chariton, 2005)

Univariate measurements were made of the biota sampled from four locations along a poly-metallic gradient within Lake Macquarie in 2000 and 2003 (Figs. 7.4 and 7.5). Contamination was least at Nords Wharf. It was postulated that the decline in fauna at Nords Wharf between 2000 and 2003 was the result of a natural phenomenon (such as unseasonal and prolonged rainfall).

In both figures, blue bars = year 2000, red bars = year 2003, CB = Cockle Bay, WB = Warners Bay, KB = Kooroora Bay, NW = Nords Wharf.

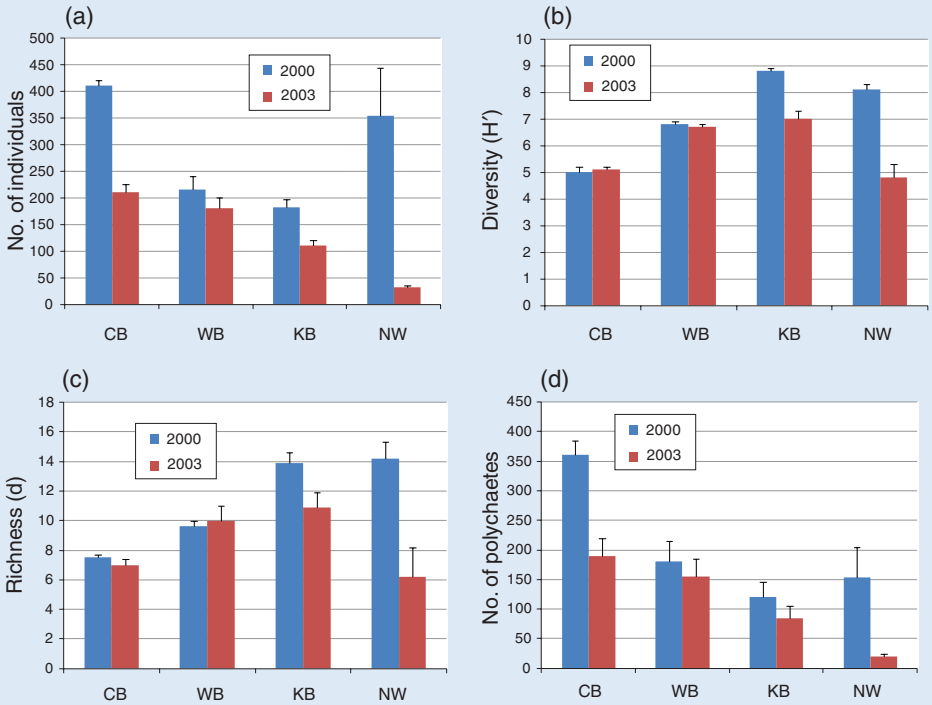

Figure 7.4. (a) No. of individuals, (b) Shannon-Wiener index of diversity (H'), (c) taxa richness and (d) number of polychaetes.

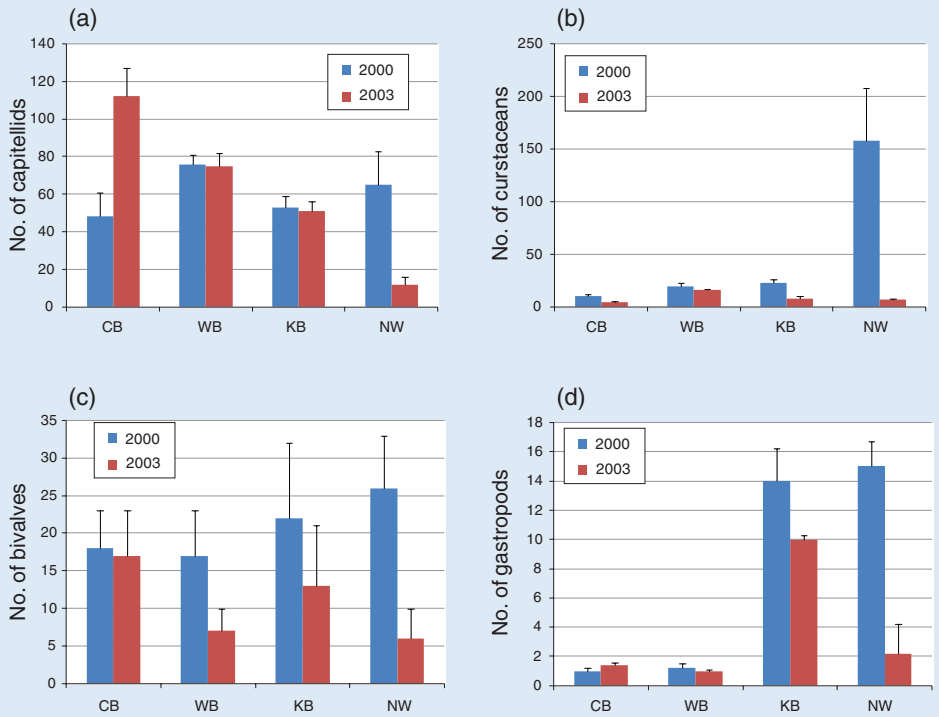

Figure 7.5. No. of (a) capitellid polychaetes, (b) crustaceans, (c) bivalves and (d) gastropods.

community level. However, several indices together may provide pertinent information on how community structure may be being modified by contaminants or other stressors.

Although commonly used, univariate measurements are much criticised because of the loss of information that occurs when assemblages are reduced to a series of variables (Matthews *et al.*, 1996). However, if founded on ecological information and theory, univariate analyses can provide additional relevant information that may add weight in discriminating between impacted and reference locations. A comprehensive review of the various metrics is beyond the scope of this section, but useful papers on the topic include those by Washington (1984), Rapport *et al.* (1985), Schindler (1987), Gray (1989), Keough and Quinn (1991) and Warwick (1993).

7.7.2 Multivariate measurements

In the past decade, there has been a substantial increase in the use of multivariate techniques in benthic contaminant studies. This has been primarily driven by an increase in the processing power of personal computers, increased access to statistical packages for analysing and interpreting benthic communities, and general acceptance of these techniques by the scientific community.

In contrast to univariate approaches, multivariate techniques do not require the data to be reduced to a single variable; rather, comparisons are made between two or more locations by quantifying the similarities (and differences) in their taxa and their relative abundances. As a result, multivariate approaches can capture and reflect differences in whole assemblages. There are numerous multivariate approaches that can be used in the analysis

and interpretation of benthic assemblages. In this section, the application of multivariate techniques is illustrated using benthic community and environmental data from five synthetic estuaries. The first technique discussed here is ordination, to visualise the similarities or dissimilarities of the ecology, and compare the biological compositions of the five estuaries. Subsequent analyses focus on exploring the relationships between the biotic and environmental (abiotic) data. This section uses the same synthetic datasets (biological and environmental data from five estuaries) in all examples, but because the data are synthetic the findings should not be interpreted literally.

Non-metric multidimensional scaling (nMDS) is one of the most commonly-used ordination techniques; it is conceptually simple to understand and is provided in numerous statistical packages including Primer (PRIMER-E, 2001, 2008), Canoco 5 (Ter Braak and Šmilauer, 2012) and the VEGAN package in R (R Development Core Team, 2013). An nMDS plot is a means of visualising a similarity matrix, with the output presented as either a 2- or 3-dimensional ordination 'map' in which samples that are more similar to each other are positioned closer than those that are less similar. In the example in Fig. 7.6, it can be inferred that the four samples from Estuary 1 are more similar to each other than they are to the samples from the other estuaries. The ordination map also suggests that samples from Estuaries 2, 3 and 5 are more similar to each other than samples taken from either Estuary 1 or Estuary 4. Samples from Estuaries 2, 3 and 5 are relatively close together and visually overlap, and it is not possible to determine whether the samples from these estuaries differ significantly from each other. Hence, nMDS does not test for difference between samples, but rather provides a graphical representation of the data that can be useful in gaining an understanding of how samples relate to each other, respond to environmental variables, or vary over time (if time series data are being analysed).

Although the graphical representation provided by nMDS is intuitive, the complex number of multivariate relationships that occurs in a large dataset, and the reduction of this information down to a 2- or 3-dimensional ordination map, can distort the spatial relationships between samples on the ordination map. Consequently, both increasing the size of the datasets and reducing the dimensions of the ordination will increase the level of distortion. In most statistical packages, the level of distortion is calculated as a measure of 'stress', with a lower stress value indicating a more accurate representation of the spatial relationships on the ordination map, thereby increasing the power of the interpretation. In Fig. 7.6, a stress value of 0.11 indicates that the ordination map provides an acceptable representation of the spatial relationships between the samples. As a guide, a more conservative interpretation should be applied in interpreting 2-dimensional ordination maps with stress values above 0.2, and those with stress values exceeding 0.3 provide arbitrary spatial relationships between the samples (Clarke and Warwick, 1994).

A number of alternative ordination techniques are complementary to nMDS. These include Principal Coordinate Analysis (PCO) which, like nMDS, is flexible, as it can be derived from a range of distance measurements. In contrast to nMDS, which focuses on preserving the rank order of dissimilarities for an *a priori* defined number of dimensions (that is, 2 or 3 dimensions), PCO is a projection onto axes that minimises residual variation in the space of the chosen distance measure (Anderson *et al.*, 2008). As such, PCO axes are quantitatively scaled (Fig. 7.7). Like nMDS, PCO is robust to outliers and has excellent distance-preserving properties.

The nMDS technique is generally recommended rather than PCO, but both approaches generally produce similar findings (Anderson *et al.*, 2008). In some cases, for instance

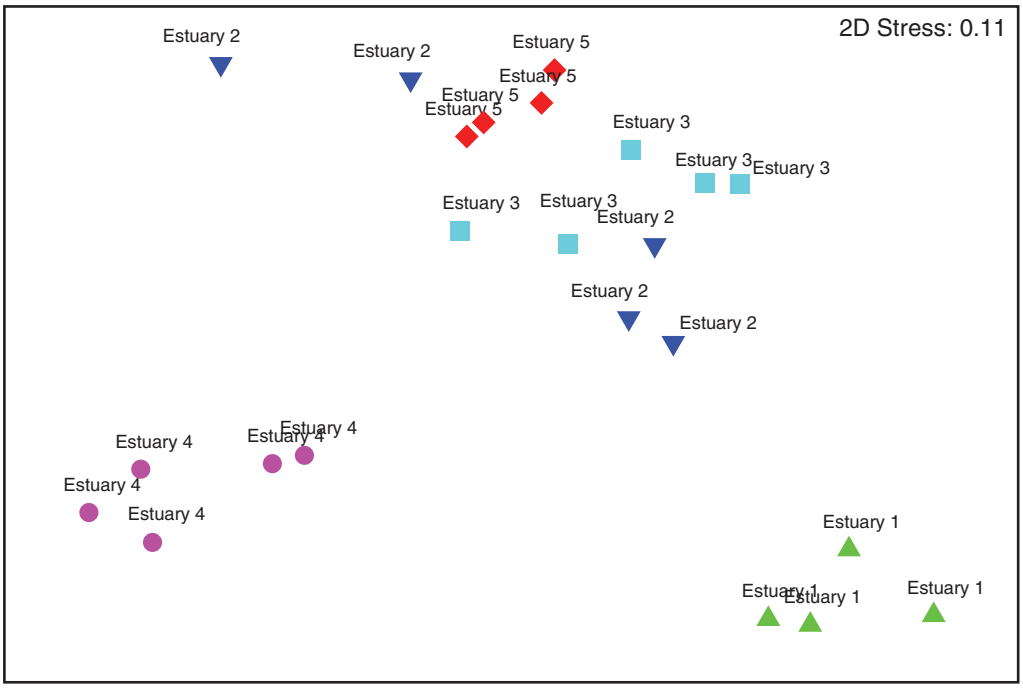

Figure 7.6. An ordination plot derived from non-metric Multidimensional Scaling Analysis (nMDS) of benthic communities sampled from five fictitious estuaries.

where all within-group dissimilarity is greater than between-group similarity, nMDS can be uninformative. In such cases, using PCO may prove more useful.

Ordination techniques, including nMDS and PCO, are visualisation tools and do not quantify the variance among or within the samples, which is traditionally measured by analyses of variance (ANOVAs). Consequently, additional analysis is required to establish whether there are compositional differences between the treatments (estuaries, in this case). The most commonly used approach for doing this is Permutational Multivariate Analysis of Variance (PERMANOVA), which is available commercially in the Primer 6+ software package (PRIMER-E, 2008). PERMANOVA can be viewed as a multivariate technique which is analogous to ANOVA, and thereby can employ the same design layouts commonly used in ANOVAs, including nested designs.

Using the same fictitious estuary dataset presented in the nMDS/PCO ordinations, PERMANOVA can be performed to examine whether there are significant differences in the biological compositions of the five estuaries. As expected, the results of the PERMANOVA confirm that there is a significant difference (PERMANOVA: Psuedo-$F = 5.71$, $P = 0.01$). In common with an ANOVA, pair-wise tests are then performed to identify where these differences occur. As indicated in Box 7.3, pair-wise testing finds all estuaries contain significantly ($P_{(perm)} \leq 0.05$) different compositions. In addition, the PERMANOVA provides information regarding the average distances between the estuaries and within the estuaries. In agreement with the nMDS plot, the distances within estuaries – that is, the aggregation of the sites from each estuary (range 42.9–57.6) – is smaller than the distances between estuaries, with the most widely dispersed sites coming from Estuary 2. The findings also numerically support the nMDS in that the most

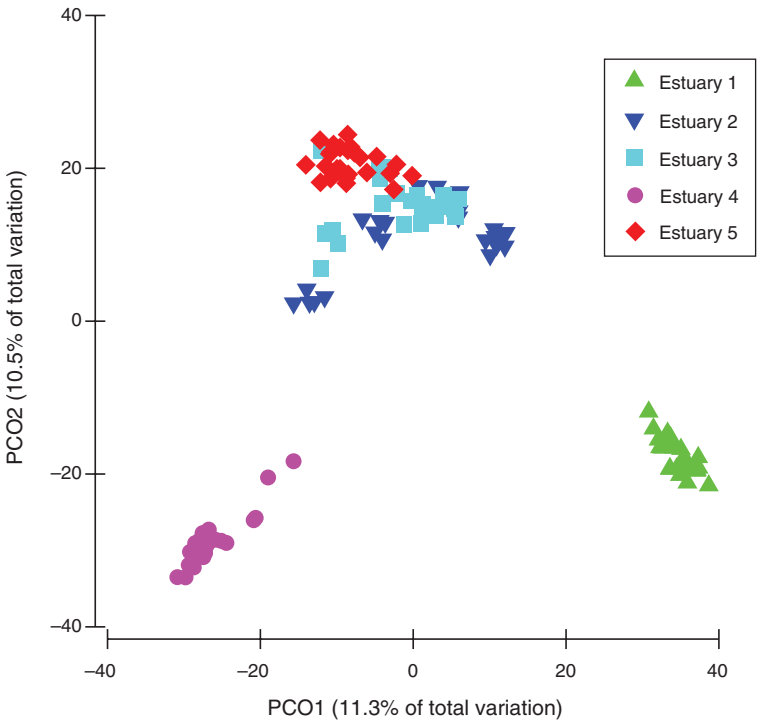

Figure 7.7. An ordination plot derived from Principal Coordinate Analysis (PCO) of benthic communities sampled from five fictitious estuaries.

dissimilar (average mean distance) estuaries are Estuary 1 and Estuary 4 (average distance = 74.7), with the less dissimilar estuaries being Estuaries 2, 3 and 5 (average distance range 59.6–69.8).

The recommended alternative to PERMANOVA is Analysis of Similarities (ANOSIM), a function in the Primer software package or freely available via the 'Adonis' function within the R package VEGAN. It is worth noting that while the pair-wise comparisons test employed in ANOSIM and PERMANOVA may appear to be analogous to univariate multiple comparisons-of-means tests, such as Tukey's and Student–Newman–Keuls, there is one distinct difference that must be considered. In the univariate tests, the effect of performing multiple tests (pair-wise Type I error rates) – that is, the accumulation of errors occurring from each test – is corrected to varying degrees within the test's algorithm, but this is not possible under the constraints of permutation tests. Consequently, either an increase in sample replication or a less pragmatic view of significance is required when interpreting the results from pair-wise comparisons produced by ANOSIM and PERMANOVA. Although correctional tests are available that can be used to adjust the *P* value in significance tests (for example, Bonferroni's test or correction), they can produce very conservative levels of significance.

7.7.3 Indicator analysis

A major interest for many ecologists is whether there are species, or groups of taxa or species, which are indicative of a particular treatment. These taxa are commonly referred to as 'indicator taxa', and are important because they: (i) may provide information on the ecological integrity of a system, (ii) reflect key shifts in composition change over time or

Box 7.3: Summary of PERMANOVA results from the comparisons of the five estuaries

Summary of pair-wise tests between estuaries

Groups	*t*	*P*(perm)
Estuary 1 *v.* Estuary 2	2.174	0.009
Estuary 1 *v.* Estuary 3	2.604	0.008
Estuary 1 *v.* Estuary 5	2.835	0.011
Estuary 1 *v.* Estuary 4	3.099	0.007
Estuary 2 *v.* Estuary 3	1.682	0.010
Estuary 2 *v.* Estuary 5	1.685	0.008
Estuary 2 *v.* Estuary 4	2.366	0.004
Estuary 3 *v.* Estuary 5	1.953	0.007
Estuary 3 *v.* Estuary 4	2.706	0.009
Estuary 5 *v.* Estuary 4	2.832	0.011

Average distance (Bray–Curtis) between estuaries and within estuaries (bracketed values)

	Estuary 1	Estuary 2	Estuary 3	Estuary 4	Estuary 5
Estuary 1	(46.9)				
Estuary 2	70.1	(57.6)			
Estuary 3	70.6	62.7	(48.2)		
Estuary 4	74.7	71.1	69.2	(42.9)	
Estuary 5	73.1	61.7	59.6	69.8	(46.4)

space, and (iii) provide inference regarding the diversity of other taxa or communities within a system (De Cáceres *et al.*, 2010).

While several approaches are available for examining the relative contributions of individual taxa to the separation of treatments – for example, Primer's SIMPER function – this procedure is only applicable to Bray–Curtis dissimilarities. Furthermore, SIMPER is not suitable for very large presence/absence metabarcoding datasets, because the analysis often results in an extensive number of taxa which only contribute to a minute fraction of the total average dissimilarity. In contrast, 'Indicator Analysis' aims to identify taxa or groups of taxa that are indicative of a particular treatment(s), rather than the taxa that contribute to the dissimilarities between treatments. Indicator analysis can be performed in R using the package 'Indicspecies' or using the 'indicator species' function in the commercial software package PC-Ord (PC-ORD, 1999). During indicator analysis, an indicator value is produced from two components: A (specificity), which is the probability of the taxa being observed in a specific treatment; and B (fidelity), the probability of finding the taxa in all sites belonging to this treatment. For example, a taxon with a perfect indicator value = 1 would be only found in one treatment (A = 1) and would be observed in all samples associated with that treatment (B = 1). Indicspecies is flexible in that it enables users to look at combinations of taxa as well as combinations of treatments. Because of the

large number of tests performed to examine the association of each taxon with its associated treatment, it is strongly recommended that the 'signassoc' function in Indicspecies be used to correct *P*-values for multiple testing.

7.7.4 Linking benthic communities to environmental variables

The primary objective of survey studies is to examine relationships between benthic assemblages and concentrations of contaminants. As previously emphasised, this approach does not prove causality, but rather assists in identifying contaminants and other variables which correlate with changes in benthic assemblages. While a wide range of statistical tools are available to explore the relationships between communities and environmental variables, the focus here is on constrained ordination techniques.

The most rudimentary approach for illustrating relationships between environmental variables and benthic assemblages is by overlying nMDS ordination maps with measurements of suspected correlating environmental variables. While this approach may provide a broad overview, visualisation is limited to one variable at time. Alternatively, all environmental variables of interest can be transposed (overlaid) onto the nMDS or PCO ordination plot (Fig. 7.8). As indicated in this diagram, copper, lead and turbidity are more elevated in Estuary 4 than the other estuaries. In the case of Estuary 2, the overlying waters of the sites have higher pH and greater electrical conductivity.

A more formal approach for examining the correlative relationship between abiotic and biotic matrices is BIOENV in Primer 5 (PRIMER-E, 2001). BIOENV performs series rank correlations between the biotic and abiotic datasets, producing a list of the best correlations. This approach is advantageous in that it not only identifies the best correlate, but also the best combination of correlates. Before the BIOENV procedure is performed, it is critical that highly correlated ($r = 0.95$) variables are removed because these will affect the

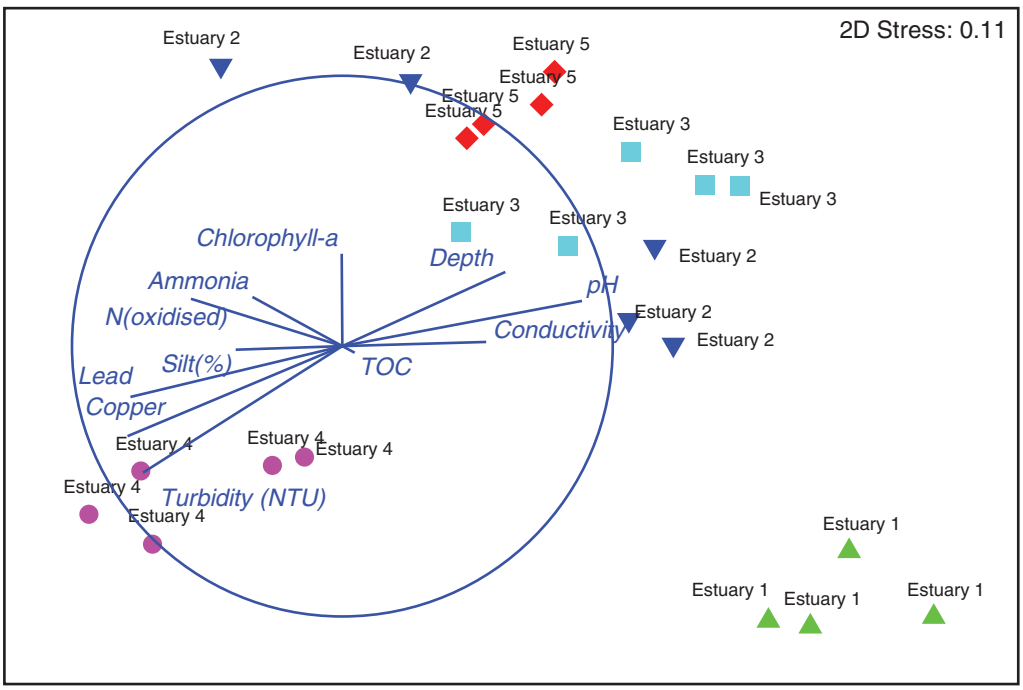

Figure 7.8. nMDS ordination plot (derived from Fig. 7.6) with the environmental variables associated with the five fictitious estuaries superimposed as vectors.

procedure. In the example provided, the variables are highly correlated, being often derived from the same source, in this case a sewage treatment plant. The production of draftsman's plots (displays of multivariate data that plot the values for each variable against the values for each of the other variables; Manly, 2004) and the output from Pearson correlation coefficients can be used to identify highly correlated variables and variables that may require transformations. Table 7.2 shows the results from a BIOENV procedure produced on the same data illustrated in Fig. 7.8. The results show that collectively five variables (ammonia, particulate lead, pH, phosphorus (reactive), and particulate copper) show the strongest correlation (r = 0.542) with benthic composition. However, similar results are also found when only lead and pH are included, emphasising the correlations between many of these variables.

The methods previously described relate the results of an ordination of biological data (for example, relative differences among samples as seen in an ordination plot) to particular environmental variables, either through a graphical approach such as bubble plots or a statistical approach such as BIOENV. In both approaches, the environmental data are not taken into account for the ordination of the biological data. This is called an unconstrained or indirect analysis.

An alternative approach, which is strongly recommended, is to use constrained or direct analyses. Constrained analyses directly integrate the environmental data into the analysis, enabling all species in the dataset (via similarity/dissimilarity matrix) to be examined in terms of their relationships with one or more of the environmental variables. For environmental surveys and manipulation experiments, these approaches have several powerful features which can greatly assist in clarifying the complex relationships which often underpin these studies. First, constrained analyses allow for the partitioning of the total variance of the biological data into independent components (for example, space, time and environmental influences) and an undefined or stochastic component (Borcard *et al*., 1992) (Fig. 7.9). From a risk assessment perspective, partitioning or partial analysis enables the investigator to identify the amount of variance in the community data which is explained solely by ecological variables, and solely by contaminants, and shared by ecological variables and contaminants, as well as the proportion of the variance that is unexplained (Chariton *et al*., 2010b) (Fig. 7.9). Furthermore, permutation methods can be used to statistically test the relationships between one or more explanatory variables (environmental variables).

Table 7.2. Summary of BIOENV results produced from Primer showing the top 10 combinations of environmental variables which correlate with community composition

No of variables	Correlation	Selected variables
5	0.542	Ammonia, lead, pH, phosphorus (reactive), copper
4	0.541	Lead, pH, phosphorus (reactive), copper
4	0.54	Ammonia, lead, pH, copper
3	0.54	Lead, pH, copper
4	0.537	Ammonia, lead, pH, phosphorus (reactive)
3	0.536	Lead, pH, phosphorus (reactive)
3	0.534	Ammonia, lead, pH
2	0.534	Lead, pH
4	0.532	Ammonia, pH, copper
3	0.555	pH, phosphorus P (reactive), temperature
5	0.553	Total organic carbon, conductivity, pH, temperature

There are various types of constrained analyses, including Canonical Correspondence Analysis (CCA) and Distance-based Linear Models (DISTLM), available via the statistical packages Canoco 5, Primer 6+ and various R packages, including VEGAN. These methods have been described in detail in the literature (Ter Braak, 1995; Legendre and Legendre, 1998; Legendre and Anderson, 1999; Šmilauer and Lepš, 2014). There are examples where these techniques have been applied to investigations of relationships between contamination and benthic communities (Rakocinski *et al*., 1997; Morrisey *et al*., 2003; Chariton *et al*., 2010a; Dafforn *et al*., 2014), and others have examined relationships between benthic communities and non-contaminant variables (Ysebaert and Herman, 2002).

The next example further illustrates the application of constrained analyses, by re-examining the relationships between biota and environmental variables from the five previously examined fictitious estuaries. This example shows the use of DISTLM (distance-based linear models) with the ordination plot of the fitted data produced using the distance-based redundancy analysis (dbRDA) function in Primer 6+. There are various ways to specify the order in which the environmental variables are built into the model, and in this example the 'all specified' option is chosen, which simply orders the variables as they appear on the original environmental data matrix.

The primary outputs of interest from DISTLM are the summaries of the 'marginal' and 'sequential' tests. Marginal tests examine the relationships between the environmental variables and the biotic similarity matrix individually; that is, not taking into account the other variables. As illustrated in Box 7.4, with the exception of chlorophyll-*a*, there are significant relationships ($P \leq 0.05$) between all measured environmental variables and biological composition. In this case, the largest amount of variability (18.6% or 0.186) is being explained by particulate copper. However, as previously stated, in cases such as this example, multiple environmental variables may be influencing biotic composition simultaneously, and consequently an amount of variation will undoubtedly be shared among the variables. The sequential test (Box 7.4) provides a summary of the relationships between the variables and the biotic data subsequent to sequentially fitting the variables. The results from this component show that collectively the environmental variables can explain 75.5% of the variation in the biological composition, and therefore 24.5% still remains unexplained. However, when examined collectively, only five variables (Box 7.4) are shown to significantly ($P \leq 0.05$) contribute to the variability in the biological data.

The most salient feature of the ordination plot produced from the analysis (Fig. 7.10) is clear separation along dbRDA1 (the horizontal axis) of the five sites (points represent the centroid means of the five replicates from each site) from Estuary 4. Furthermore, the direction and length of the environmental vectors for copper and turbidity suggest that the composition of the benthic communities from Estuary 4 is strongly correlated with

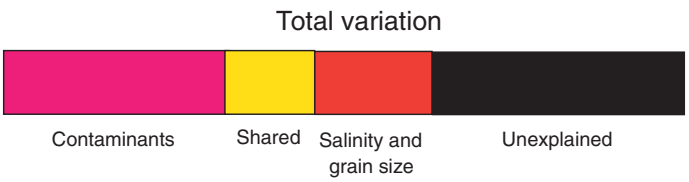

Figure 7.9. A conceptual diagram illustrating the partitioning of variation from a hypothetical study examining the responses of benthic communities to contaminants along an estuary. In this example, ~30% of the variation can be explained by contaminants and 20% by natural variables such as salinity and grain size. There is a 10% overlap between these variables because they are partially correlated. Approximately 30% of the variation in the biological dataset is unexplained.

Box 7.4: Summary of the DISTLM procedure in Primer 6+, examining the correlative relationships between benthic communities and the environmental attributes of five estuaries

Marginal tests

Variable	SS(trace)	Pseudo-F	*P*	Proportion
Total organic carbon	2552	1.194	0.257	0.054
Chlorophyll-*a*	3202	1.519	0.068	0.067
Conductivity	6850	3.542	0.001	0.144
Secchi depth	5109	2.534	0.004	0.108
Ammonia	4125	1.999	0.013	0.087
Organic N	7337	3.840	0.001	0.155
Lead	4995	2.470	0.005	0.105
Total N	7363	3.857	0.001	0.155
Dissolved oxygen	6031	3.057	0.001	0.127
pH	8230	4.406	0.001	0.173
Copper	8838	4.806	0.001	0.186
Temperature	6167	3.137	0.001	0.130
Turbidity	8538	4.607	0.001	0.180

Sequential tests[a]

Variable	R^2	SS(trace)	Pseudo-F	*P*	Proportion	Cumulative
Copper	**0.186**	**8838**	**4.806**	**0.001**	**0.186**	**0.186**
Lead	**0.266**	**3762**	**2.159**	**0.007**	**0.079**	**0.266**
Turbidity	**0.347**	**3856**	**2.363**	**0.006**	**0.081**	**0.347**
pH	**0.408**	**2907**	**1.863**	**0.012**	**0.061**	**0.408**
Chlorophyll-*a*	**0.461**	**2523**	**1.677**	**0.029**	**0.053**	**0.461**
Dissolved oxygen	0.509	**2264**	**1.554**	**0.061**	**0.048**	**0.509**
Temperature	0.555	2165	1.536	0.054	0.046	0.555
N (organic)	0.593	1847	1.340	0.142	0.039	0.593
Total organic carbon	0.624	1436	1.046	0.392	0.030	0.624
Conductivity	0.654	1443	1.055	0.412	0.030	0.654
Secchi depth	0.684	1413	1.036	0.426	0.030	0.684
Ammonia	0.713	1369	1.004	0.44	0.029	0.713
N (total)	0.736	1111	0.799	0.641	0.023	0.736
Silt content	0.755	873	0.599	0.828	0.018	0.755

[a] Bold font indicates variables contributing significantly ($P \leq 0.05$) to the variability in the biological data.

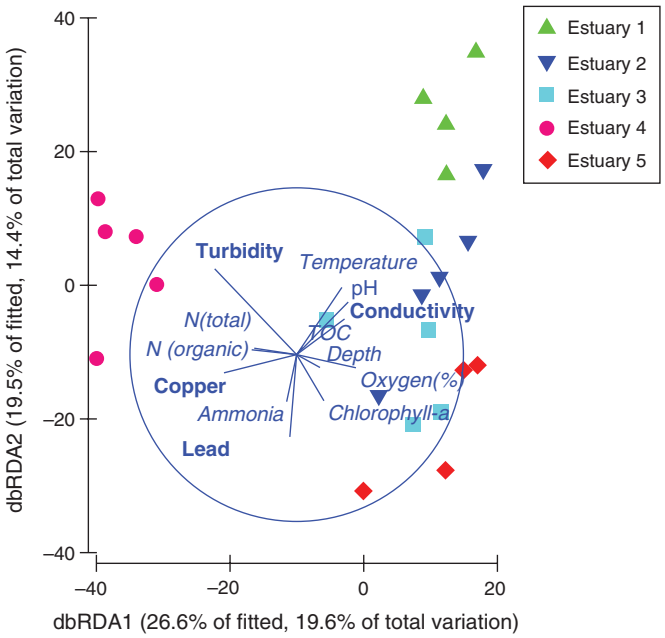

Figure 7.10. dbRDA plot derived from the synthetic biological and environmental data from the five fictitious estuaries.

these variables. Similarly, the positioning of the sites from Estuary 1 and Estuary 2 suggests a strong relationship between composition and conductivity within the estuaries, reflecting that the positions of the sites extend from the marine-influenced mouth of the estuary to freshwater-dominated sites upstream. While not illustrated in this example, triplots can be also be created, with the additional axis used to show the trajectory of key taxa; for example, the occurrence and abundance of a particular nematode species may also increase in the direction of the sites associated with Estuary 4.

7.7.5 Further exploring the community responses along environmental gradients

In addition to the commonly described approaches for examining the relationships between benthic biota and natural and anthropogenic stressors, recently several additional statistical techniques have been developed to further explore the data by identifying tipping-points, or thresholds, for species and communities to environmental variables (Baker and King, 2010; Ellis *et al.*, 2012). A particularly useful technique is the Threshold Indicator Taxa ANalysis (TITAN) in R (Baker and King, 2010). TITAN is extension of indicator analysis (see Section 7.7.3 above); instead of examining the occurrence or abundance of taxa between and within treatments, the analysis examines how each taxon responds to an environment gradient. This is performed by partitioning the data into two groups at the value of a predictor variable that maximises the association of each taxon with those either side of the partition. Using standardised z-scores, TITAN can distinguish those taxa for which the occurrences decline ($-z$ scores) or increase ($+z$ scores) along the environmental gradient. TITAN uses bootstrapping to estimate the confidence limits of the change points, as well as purity (the proportion of the replicates with the same positive or negative response), and the width of the confidence limits potentially provides information regarding the tolerances of each taxon and the community to the environmental gradient.

An example of one of the outputs provided by TITAN is provided in Fig. 7.11 using the synthetic data of the five fictitious estuaries examined in the previous section. In this example, the top ten taxa which respond negatively to increases in particulate copper (based on their $-z$ scores) are presented on the left diagram (black), and those which respond positively are presented on the right (orange). As indicated in this example, those taxa sensitive to copper ($-z$ taxa) are generally only found within a narrow range of particulate copper concentrations (x-axis) and are absent where concentrations exceed 60 mg/kg. In contrast, those taxa which respond positively to copper are observed over a wide range of concentrations, and most of these taxa are detected at particulate copper concentrations above 100 mg/kg.

In addition to this information, peak and cumulative values in $-z$ and $+z$ scores can then be used to respectively determine negative and positive community responses to the environmental variable. Evidence of a community change is estimated by examining synchronous changes in occurrences and/or abundances within a narrow range of predictor values. Collectively, the analysis infers that there is a community change point (threshold) – that is, the point where there is a maximal change in community structure. Furthermore, although the provided example explores the relationships between the biota and particulate copper, as indicated in Fig. 7.11, other co-variables are undoubtedly contributing to changes in the biological composition along this gradient. Chariton *et al.* (2015) provide a recent example of using TITAN with metabarcoded data to examine the responses of estuarine taxa to natural and anthropogenic gradients.

Although TITAN can provide information that is potentially useful ecologically, the package is currently limited to examining a single environmental variable at a time. However, scores from Principal Component Analysis (PCA) may be used to capture gradients that encompass multiple variables (for example, see Suarez-Rubio *et al.*, 2013). Alternatively, the R-package, gradientForest (Ellis *et al.*, 2012), which is an extension of the random forest approach, is suited for delineating the key environmental predictors for composition changes and identifying where compositional changes may occur along these gradients. Regardless of the approach taken, it is important to note that these techniques

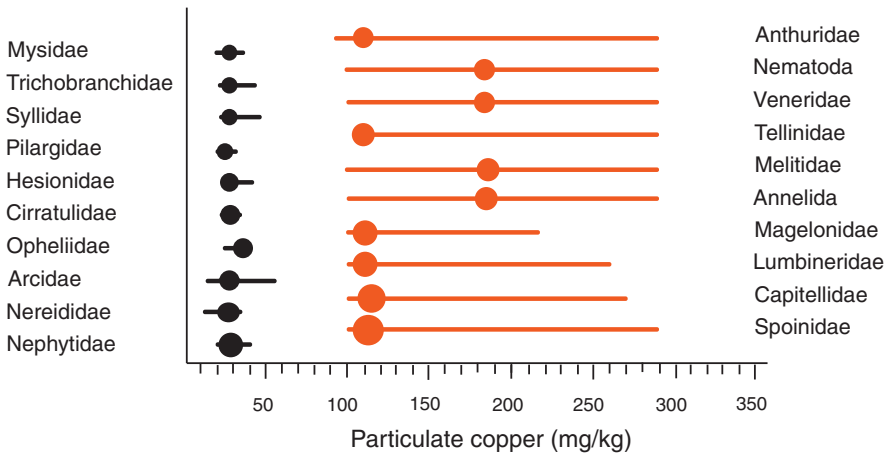

Figure 7.11. Summary of Threshold Indicator Taxa ANalysis (TITAN) results illustrating the change points and 95% confidence limits for the top 10 (highest $-z$ and $+z$ scores) taxa which respond significantly to an increase in particulate copper concentrations. The size of the points is scaled to reflect the magnitude of their response (z scores).

are not designed to replace traditional multivariate approaches. Rather, if suitable, they should be used as complementary approaches to further explore the data.

7.8 Placing benthic community studies in context

Assessment of *in situ* benthic communities can provide important information on the level of biological organisation, and over a time scale that is beyond the scope of laboratory studies. However, well-designed field studies cannot obviate the need for well-executed toxicity tests, because they are not based on the underlying experimental principles of cause and effect – with the notable exception of *in situ* bioassays (Baird *et al*., 2007). Under the sediment quality assessment framework described in Chapter 1 Section 1.3, multiple lines of evidence from a combination of chemical, biological and ecological studies are required, to increase our capacity to assess the risk associated with contaminated sediments. If no effect on benthic communities is observed using a suitable experimental design and analyses, then this would indicate that there is low risk of contaminant-induced perturbation. This does not negate the possibility that trace contaminants are affecting benthic communities; it only reduces the likelihood that these are having a significant impact under the frame of reference chosen for measuring ecological stress. Consequently, how stress is defined, measured and quantified will determine the ecological outcomes of the study.

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8

Summary

Stuart L. Simpson and Graeme E. Batley

The use of multiple lines of evidence, consistent with the integrated assessment philosophy of the ANZECC/ARMCANZ (2000) guidelines, is currently the best approach for assessing sediment quality. This can be achieved by extending the current ANZECC/ARMCANZ decision framework to include bioaccumulation and ecological assessments, or by combining these and other lines of evidence (chemistry, toxicity) in a weight-of-evidence framework. Thus, ideally, investigations should combine assessments of:

- sediment chemistry (such as exceedances of sediment quality guidelines), including contaminant bioavailability tests (for example, pore-water measurements, acid volatile sulfide tests, and biomimetic approaches for hydrophobic organic contaminants);
- toxicity testing (for example, of multiple species, varying exposure pathways, and acute and chronic endpoints such as mortality, growth, reproduction or avoidance);
- bioaccumulation or biomagnification; and
- benthic community structure (such as ecological malfunction).

Toxicity identification evaluation and other causality considerations may also be of value. The combination and interaction between lines of evidence should be considered in applying these in a weight-of-evidence framework. (For instance, particle size affects contaminant bioavailability; bioavailability test results will affect the interpretation of toxicity and bioaccumulation data.) Weight-of-evidence assessments often ultimately rely on best professional judgment, but the use of tabular decision matrices is the best approach for achieving transparency and comprehension by personnel outside the field of field of ecological risk assessment.

This handbook has described approaches for measuring the various lines of evidence. As new lines of evidence are continuing to be developed, future sediment quality assessments may also incorporate those. While a general approach has been proposed in this book, assessments frequently need to be custom-designed and lines of evidence chosen to suit the site-specific circumstances (such as site dynamics, sediment stability, groundwater flows, and fluctuating overlying water conditions).

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Appendix A: Sediment quality guideline values

Stuart L. Simpson and Graeme E. Batley

A.1 Current guideline values and recommended revisions

This appendix (Table A.1) gives guideline values from ANZECC/ARMCANZ (2000) which are still current in 2015, and revisions to the guideline values as indicated by the footnotes. In using this table, all comparisons of guideline values with test sediment concentrations should initially be made using the total contaminant concentration in the <2 mm fraction.

Table A.1. Recommended sediment quality guideline values (SQGV and SQGV-high)

Contaminant	Guideline value (SQGV)	SQGV-high
Metals[a]	(mg/kg dry weight)	(mg/kg dry weight)
Antimony	2.0	25
Cadmium	1.5	10
Chromium	80	370
Copper	65	270
Lead	50	220
Mercury	0.15	1.0
Nickel	21	52
Silver	1.0	4.0
Zinc	200	410
Metalloids[a]	(mg/kg dry weight)	(mg/kg dry weight)
Arsenic	20	70
Organometallics[b,c]	(µg Sn/kg dry weight, 1% OC)	(µg Sn/kg dry weight, 1% OC)
Tributyltin	9.0	70
Organics[b,d]	(µg/kg dry weight, 1% OC)	(µg/kg dry weight, 1% OC)
Total PAHs (sum of PAHs)[e]	10,000	50,000
Total DDT	1.2	5.0
p,p'-DDE	1.4	7.0

(continued)

Table A.1. (Continued)

Contaminant	Guideline value (SQGV)	SQGV-high
o,p'- + p,p'-DDD	3.5	9.0
Chlordane	4.5	9.0
Dieldrin[f]	2.8	7.0
Endrin[f]	2.7	60
Lindane	0.9	1.4
Total PCBs	34	280
Total petroleum hydrocarbons (TPHs)[g]	280 (mg/kg dry weight)	550 (mg/kg dry weight)

[a] Primarily adapted from the 'effects range low'/'effects range medium' (ERL/ERM) values of Long *et al.* (1995).
[b] Normalised to 1% organic carbon (OC) within the limits of 0.2 to 10%. Thus if a sediment has: (i) 2% OC, the '1% normalised' concentration would be the measured concentration divided by 2; (ii) 0.5% OC, then the 1% normalised value is the measured value divided by 0.5; (iii) 0.15% OC, then the 1% normalised value is the measured value divided by the lower limit of 0.2.
[c] Basis of revision is described in Appendix A2 of Simpson *et al.* (2013).
[d] Primarily adapted from 'threshold effects level' (TEL) and 'probable effects level' (PEL) values of MacDonald *et al.* (2000) and CCME (2002).
[e] The GV and SQGV-high values for total PAHs (sum of PAHs) are described in Appendix A3 of Simpson *et al.* (2013) and include the 18 parent PAHs: naphthalene, acenaphthylene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo(a)pyrene, perylene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(e)pyrene, benzo(ghi)perylene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene. Where non-ionic organic contaminants like PAHs are the dominant chemicals of potential concern (COPCs), the use of the 'equilibrium sediment benchmark' (ESB) approach is desirable, and is applied as outlined in Appendix A3 of Simpson *et al.* (2013), which includes a further 16 alkylated PAHs (generally listed as C1-/C2-/C3-/C4-alkylated).
[f] Where dieldrin or endrin are the major COPCs, it is recommended that ESB approaches are applied as described in Appendix A4 of Simpson *et al.* (2013).
[g] Origin described in Appendix A5 of Simpson *et al.* (2013).

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Appendix B: Preparation of contaminant-spiked sediments

Stuart L. Simpson

B.1 Introduction

The preparation of contaminant-spiked sediments is frequently undertaken for the purpose of:

(i) checking recoveries of analytes (QA/QC);
(ii) understanding the partitioning of contaminants between sediments and water in different sediment matrices;
(iii) understanding the transformation rates (degradation, volatilisation) of contaminants in different sediment matrices; and
(iv) quantifying the effects of known concentrations of contaminants in toxicity tests.

The intent of the spiking procedure is usually to achieve a uniform distribution of a particular contaminant concentration in a given sediment. However, for purposes (iii) and (iv) above, it is usually necessary to achieve environmentally realistic partitioning of the added (spiked) contaminant between the water and particulate phase, in order to proceed. The spiking procedures, as well as the properties of the contaminant and sediment, will influence the final partitioning achieved. It is therefore important to fully document the procedure used (Northcott and Jones, 2000; Simpson *et al*., 2004). Key parameters to consider reporting include:

- the chemical formula and form of the added chemical (e.g. dissolved in water or alternative solvent);
- the procedure used for mixing and homogenisation (frequency and duration of mixing);
- equilibrium period (days to months) and conditions (e.g. oxygen-free environment; temperature);
- sampling and analysis undertaken to monitor the partitioning of the added chemical;
- sediment parameters, including moisture content, pH, redox potential, organic carbon, acid volatile sulfide (AVS), particulate iron and manganese (for metals);
- pore-water constituents, including dissolved ammonia, sulfide, iron, manganese and other metals;
- pore-water contaminant concentrations.

Many of these parameters will greatly assist in understanding the partitioning and bioavailability of the added chemical and assist in interpreting results. Often it is useful if a control sediment (unspiked) undergoes the same treatment as the spiked sediments to provide an understanding of how the sediment-manipulation aspects of the procedure (for example, homogenising and equilibration) influence the sediment properties (for example, the pH, particle size distribution or AVS concentration).

B.2 Method

Wet 'slurry' spiking techniques are recommended over dry spiking, to facilitate sediment–water interactions (Landrum *et al.*, 1992; Northcott and Jones, 2000; USEPA, 2001; Simpson *et al.*, 2004). The time required to achieve adequate equilibration may range from several week to months (Simpson *et al.*, 2004; Hutchins *et al.*, 2009; Brumbaugh *et al.*, 2013; Vandegehuchte *et al.*, 2013). Dilution of a 'super spike' (a very high spiked concentration; also referred to as indirect spiking) has generally been found to require less time to achieve the desired level of metal partitioning (i.e. a range of lower concentrations of metals in pore waters) (Hutchins *et al.*, 2008; Brumbaugh *et al.*, 2013). For organics, equilibration times are often shorter than for metals (e.g. days rather than weeks; Landrum *et al.*, 1992; Northcott and Jones, 2000). With organics, it is very important to consider the hydrophobicity/solubility (often indicated from the K_{OW}), choice of organic-carrier solvent for spiking (solvent persistence, volatility, toxicity), and losses of chemical through non-target adsorption, degradation (UV light), transformation (hydrolysis) and volatilisation, along with the organic carbon content of the sediment (Ankley *et al.*, 1994; Northcott and Jones, 2000; Fuchsman and Barber, 2000; OECD, 2007, 2010).

B.2.1 Key considerations for preparing metal-spiked sediments

The following recommendations are given for the preparation of metal-spiked sediments for toxicity testing.

(i) When spiking metals into sediments, the decrease in pore-water pH caused by hydrolysis reactions may require neutralising (Simpson *et al.*, 2004; Hutchins *et al.*, 2007). Buffer solutions are not suitable for increasing the pH of sediments that are naturally well buffered, and a strong base, e.g. NaOH, will generally be required. Neutralisation of pH changes should be made a few hours after metal additions and sediment mixing, to allow natural metal precipitation, adsorption and ion-displacement reactions to occur. This will lower the formation of metal-hydroxide colloids or precipitates that may be slow to equilibrate with other sediment phases (e.g. sulfide).

(ii) Metal additions will cause the displacement of Fe(II) into the pore waters, which will subsequently oxidise and hydrolyse resulting in decreases in pH and increases in redox potential (Simpson *et al.*, 2004; Hutchins *et al.*, 2008):

$$Fe^{2+} \rightarrow Fe^{3+} + e^{-}$$
$$Fe^{3+} + 3H_2O \rightarrow Fe(OH)_3 + 3H^{+}.$$

(iii) High Fe(II) concentrations (>100 mg/L) in the pore waters may affect the bioavailability of other pore-water metals. Increasing the pH to greater than pH 7.5 and then mixing will result in the rapid precipitation of most pore-water iron.

(iv) As sediments are generally anoxic a few millimetres below the sediment–water interface, air penetration into sediments should be minimised. This can be done by using deoxygenated waters for preparing metal-spike solutions, and an inert gas

(e.g. nitrogen) to occupy the bottle headspace during mixing (Simpson *et al*., 2004; Hutchins *et al*., 2008; Brumbaugh *et al*., 2013). Low oxygen conditions will reduce the oxidation of Fe(II) that causes the pH to decrease (as described above).

(v) Equilibration rates of pore-water metals vary considerably and are dependent on sediment and metal properties. Typically, equilibration times of two weeks or longer will be necessary to achieve adequately low and stable pore-water metal concentrations. Pore-water metals will equilibrate faster in sediments with high concentrations of metal-binding sites (e.g. iron, organic matter and sulfide) and large surface areas (fine, silty sediments) than in sandy sediments with low binding capacities. Equilibration rates will be slower in oxic/sub-oxic surface sediments than in sulfidic sediments. For many situations, equilibration times of months to years may result in more environmentally realistic partitioning of metal between the dissolved and various particulate phases. Longer equilibration times will enable a greater portion of initial metal precipitates to transform from the very labile forms (e.g. dilute-acid extractable metals) to much less labile forms that are less easily extracted (chemically) and less bioavailable.

(vi) Serial dilution of sediment spiked with a very high concentration of the desired metal (the indirect spiking or 'super spike' method) has been found to be a faster way of achieving low concentrations of metals in pore waters (Hutchins *et al*., 2009; Brumbaugh *et al*., 2013; Vandegehuchte *et al*., 2013).

(vii) AVS in sediments bind metals strongly, and the AVS concentration may be used to estimate when pore-water concentrations of metals should be negligible (very low, such as μg/L range) (Berry *et al*., 1996). The formation of AVS occurs slowly during the equilibration of sediments, and the addition of sulfide may be useful if metal sulfide formation is desired (Gonzalez, 1996; Simpson *et al*., 2012).

(viii) Sediments equilibrate more slowly when incubated at 4°C than at room temperature (18–25°C) resulting in high pore-water concentrations of all metals (Simpson *et al*., 2004). Disturbances to sediments during sample manipulation will cause Fe(II) oxidation and losses of metals from pore waters through adsorption to fresh iron hydroxide precipitates. This may be used to advantage to keep metal concentrations low in pore waters. Metal-spiked sediments should be equilibrated at room temperature.

(ix) Additions of metals to sediments and incubation (conditioning) of sediments may change bacterial and algal populations. Consideration should be given to how these changes will affect contaminant bioavailability and food sources for the test species. The addition of clean food sources may be required following the equilibration of spiked sediments.

(x) It is recommended that pore-water metal concentrations, pH and redox potential be measured at regular periods during sediment equilibration and at the beginning and end of tests, and reported when publishing toxicity test results.

(xi) The homogeneity of the spiked compound should be determined and reported.

(xii) Measurements of metals in overlying waters should be made following transfer of sediments to test containers and during organism exposure experiments, because this metal flux may represent a major source of metal toxicity for many organisms (Roman *et al*., 2007; Simpson and Batley, 2007; Besser *et al*., 2013; Campana *et al*., 2012, 2013; Vandegehuchte *et al*., 2013). Fluxes of metals from the sediments to the overlying waters will provide valuable information on the disturbances test organisms cause to the equilibrated metal-spiked sediments. Changes to metal concentrations in overlying waters will affect the responses and exposure pathways of test organisms.

B.2.2 Key considerations for preparing organic-compound-spiked sediments

The following key recommendations are given for the preparation of sediments spiked with organic compounds for toxicity testing.

(i) Most of the factors that are important for metal-spiked sediments (listed above) are also important for organic-spiked sediments (Fuchsman and Barber, 2000; Northcott and Jones, 2000; USEPA, 2001; OECD, 2010).
(ii) Samples should be maintained under field moisture conditions and not dried and rewetted during spiking procedures.
(iii) Careful consideration should be given to the choice of organic carrier solvent for spiking (solvent persistence, volatility, toxicity).
(iv) The amount of carrier solvent used should be kept to a minimum and be the same for controls and all spike concentrations. Spiking methods that involve coating the spiking container with the organic compound followed by volatilisation of excess solvent are useful for minimising effects of carrier solvents (Cole *et al*., 2000; Fuchsman and Barber, 2000; Northcott and Jones, 2000).
(v) Mixing should begin immediately after spiking and be sufficient to achieve homogeneity but minimised to prevent unnecessary disturbances to sediment properties. The homogeneity of the spiked compound should be determined.
(vi) If possible, the persistence of the carrier solvent in the sediment should be determined and effects on biota evaluated.
(vii) Many PAHs degrade under ultraviolet light and the degradation products may exhibit greater toxicity than the original compounds (Ankley *et al*., 1994); therefore, spiking sediments with PAHs, and PAH equilibration with sediments, should occur in the dark or at least under low light.
(viii) Short equilibration times (e.g. 24–72 h) may be suitable for organic compounds with low octanol:water partition coefficients (K_{OW}). Organic compounds with a high partition coefficient might require months to establish equilibrium (Landrum *et al*., 1992). Organic contaminants may undergo microbiological degradation, given time, which may be another important factor in the choice of an appropriate equilibration period.
(ix) Measurements should be made of the amount and type of organic carbon in the sediments because organic carbon will greatly affect the rates of equilibration and partitioning of organic spikes (Gustafsson *et al*., 1997, 2001; USEPA, 2003, 2012).

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Appendix C: Acid Volatile Sulfide (AVS) analysis

Stuart L. Simpson

C.1 Introduction

The bioavailability of metals in sediments is heavily influenced by sediment properties and the presence of substances that strongly bind metals (see Chapter 3, Section 3.6.1). As described in Chapter 3, the naturally occurring amorphous monosulfides FeS and MnS readily react with several dissolved metals to form metal-sulfide precipitates. The major sulfide-forming metals include Ag, Cd, Cu, Ni, Pb, and Zn (Rickard and Morse, 2005). The reactive forms of sulfide can be measured as acid volatile sulfides (AVS). For sediment quality assessments, the AVS concentration is defined operationally as the sulfide released from the sediment with 1 M HCl (Allen *et al.*, 1991; USEPA, 2005). The metals that are released from the same treatment are defined as simultaneously extracted metals (SEM) – the process is analogous to that used for dilute-acid extractable metal (AEM) analyses, as noted in Chapter 3 Section 3.6.1. The use and limitations of the AVS-SEM equilibrium partitioning (EqP) theory for predicting the risk of adverse effects from the metals Ag, Cd, Cu, Ni, Pb, Zn, and Cr(III) are well described by USEPA (2005) and Simpson *et al.* (2013).

In this appendix, procedures are provided for two methods that can be used for assessing the AVS concentration of sediments: (i) the purge-and-trap method described by Allen *et al.* (1991), and (ii) the rapid method described by Simpson (2001). AVS is operationally defined by the extraction procedure, and differences in methodologies may potentially provide very different results. It has been observed that there can be great variability among laboratories analysing the same samples for AVS (Hammerschmidt and Burton, 2010). It is important to note that the methods used for AVS analyses in soils frequently use more concentrated HCl (e.g. 6 M HCl), resulting in a different operationally defined sulfide fraction (i.e. higher measured metal concentrations), which is not equivalent to the AVS considered for most sediment quality assessments. Other factors influencing both the AVS and SEM results include the low solubility of some Ag, Cu and Ni sulfide phases in 1 M HCl (Cooper and Morse, 1998; Simpson *et al.*, 1998), and interference caused by dissolution of minerals that release Fe(III) to solution (e.g. FeOOH, $Fe(OH)_3$). Such release may cause an underestimation of AVS concentrations (Simpson *et al.*, 1998; Hsieh *et al.*, 2002).

C.2 'Purge-and-trap' AVS method

The 'purge-and-trap' method is applicable to sediments having up to 300 mmol AVS/kg (dry weight) (frequently reported with the units μmol/g). The detection limit is ~0.01 mmol/kg when using the methylene blue colorimetric method for determining sulfide concentrations. Because sulfide is unstable in the presence of oxygen it is necessary to protect samples from exposure to air. It is recommended that samples be frozen immediately after collection, and that all subsequent manipulations (including thawing) are carried out in a nitrogen or argon atmosphere.

C.2.1 AVS evolution and H_2S trapping

The apparatus and procedure are based on the method recommended by the USEPA (Allen *et al.*, 1991, 1993). The apparatus consists of a 250 mL round-bottom flask with a sidearm and two 15 mL impingers with non-fritted inlets. Sediment is weighed into the round-bottom flask and acid is introduced via the side arm. The apparatus is connected via a flow controller to a nitrogen line that purges the evolved hydrogen sulfide from the reaction flask to the traps.

The following modifications have been made to the method described by Allen *et al.* (1991).

- The volume of H_2S trapping solution (0.5 M NaOH) has been reduced from 80 mL to 10 mL without any deterioration in system performance.
- The nitrogen flow rate, 20 mL/min, used by Allen *et al.* (1991) was found to give unacceptably low recoveries, suggesting that flow rate may be system dependent. The nitrogen flow rate has been re-optimised at 250–300 mL/min which gives around 100% recovery from tests conducted using estuarine sediments that have 150 mmol AVS/kg.
- Tests indicate that standard high purity 'cylinder' nitrogen gives acceptable performance.

Instrumentation and apparatus

- A visible spectrophotometer capable of measuring absorbance at 670 nm.
- Inductively coupled plasma atomic emission spectrometer (ICPAES) suitable for the determination of SEM.
- Magnetic stirrer and Teflon® coated stirring bars.
- Nitrogen-filled glove box and argon purged fish tank for sample handling and manipulation.
- Drying oven.
- Top-loading balance capable of weighing 0.01 g.
- General laboratory glassware.
- Laboratory paraffin film (e.g. Parafilm® M).

Reagents

All water and reagents must be free of dissolved oxygen and sulfides. Only deionised water (e.g. Milli-Q® water) that has been purged by nitrogen for at least three hours should be used.

H_2S trapping solution, 0.5 M NaOH

Trapping solution consists of 20 g sodium hydroxide (NaOH) in 1 L of deoxygenated deionised water, and is prepared daily.

Methylene blue reagent (MBR)

Component A: Concentrated sulfuric acid (660 mL) is added to 340 mL of deoxygenated deionised water. After the solution cools, 2.5 g N-N-dimethyl-p-phenylenediamine oxalate is added (store in a freezer) and mixed until it has dissolved. (Note: replace N-N-dimethyl-p-phenylenediamine oxalate every 6 months.)

Component B: Ferric chloride hexahydrate ($FeCl_3.6H_2O$) (5.4 g) is dissolved in 100 mL concentrated hydrochloric acid and diluted to 200 mL with deionised water.

The methylene blue reagent is prepared by mixing components A and B.

This reagent should be stored in an amber glass bottle. It is stable for at least one month.

Hydrochloric acid, 6.0 M

Concentrated hydrochloric acid (500 mL of 12 M HCl, analytical reagent grade or better) is diluted with deionised water to 6 M HCl in a 1 L volumetric flask. This solution is deaerated as required by bubbling deoxygenated nitrogen through for at least 30 min before use. If ICPAES blanks are unacceptable, a better grade of acid should be used. (This solution is diluted to 1 M HCl in the AVS-SEM extraction.)

Sulfuric acid, 0.5 M

Concentrated sulfuric acid (28 mL of 18 M H_2SO_4) is diluted with deionised water to 0.5 M H_2SO_4 in a 1 L volumetric flask.

Starch indicator

Soluble starch (1.0 g) is dissolved in 100 mL hot water.

Standard sodium thiosulfate solution, 0.025 M

A 0.1 M sodium thiosulfate solution is prepared by diluting 250 mL of 0.2 M standard sodium thiosulfate (e.g. Volucon, BDH ampoule) with deionised water to make 500 mL (or as specified in the instructions). This solution is then further diluted to prepare a working stock solution of 0.025 M by transferring 250 mL to a 1 L volumetric flask and filling to1 L with deionised water.

Standard iodine solution, 0.025 M

A 0.025 M iodine solution is prepared by diluting 250 mL of 0.1 M standard iodine solution (e.g. Volucon, BDH ampoule) with deionised water to make 1 L (or as specified in the instructions).

Sulfide stock solution, ~0.05 M

The sulfide stock solution is prepared by weighing out ~5 g of $Na_2S.9H_2O$ (Sigma, ACS), rinsing it quickly (to remove adsorbed impurities) and dissolving it in 500 mL of deoxygenated deionised water. This concentrated sulfide stock solution should be stored in the nitrogen-filled glove box.

The sulfide stock solution is standardised by adding an excess of 0.025 M iodine solution, then titrating the excess iodine with 0.025 M thiosulfate solution using starch as an indicator (APHA/AWWA/WEF, 2012). The sulfide stock solution should be restandardised every 2 weeks. It has a storage life of up to 6 months if kept in the nitrogen-filled glove box.

For the standardisation, 10.0 mL of 0.025 M standard iodine solution is pipetted into each of two 125 mL Ehrlenmeyer flasks.

Sulfide stock solution (2.0 mL) is pipetted into one flask and 2.0 mL of deionised water is pipetted into the other flask as a reagent blank.

To each flask, 5.0 mL of 6 M HCl is added. The flasks are swirled slightly, then covered with laboratory paraffin film and placed in the dark for 5 min.

Each flask is titrated with the standard 0.025 M thiosulfate solution, adding soluble starch indicator when the yellow iodine colour fades. The end point is reached when the blue colour disappears.

The sulfide concentration is calculated as shown below.

AVS distillation procedure

- The frozen sample is placed in a nitrogen-filled glove box and allowed to thaw overnight.
- The stoppered reaction flask is pre-weighed on a top-pan balance (with magnetic flea).
- Deoxygenated deionised water (100 mL) is added to the reaction flask, using a measuring cylinder.
- Using a clean 25 mL syringe, 10 mL 0.5 M NaOH trapping solution is measured into each of two H_2S trapping tubes. The tubes are fitted in position and secured with rubber bands.
- The reaction flask is fitted to the AVS system and bubbled with nitrogen for 2 min at 280 mL/min (or a flow rate of 2–3 bubbles a second); making sure the flow rate is not so high that the 0.5 M NaOH trapping solution is displaced from the tubes.
- The analysis sample is homogenised in the nitrogen-filled glove box by mixing it using a plastic-coated spatula.
- About 25 g of wet sample is transferred to a pre-weighed 30 mL polycarbonate vial. The weight is recorded and then the vial is placed into a drying oven (110°C) and left there overnight. The vial is transferred to a desiccator and allowed to cool to room temperature. The vial is reweighed including the dried sediment sample, and the dry weight/wet weight ratio is calculated and used to calculate the dry weight of sediment used in the AVS measurement.
- Wet sediment (between 1 g and 2 g) is transferred to a strip of laboratory paraffin film on a tared top-pan balance. If the sample has a high water content, it is transferred to a small plastic weighing tray. The wet weight of the sample is recorded. Large masses of calcareous samples should be avoided because they will cause excessive frothing.
- The weighed sample is transferred *with a minimum of delay* to the reaction flask. The laboratory paraffin film may be inserted through the side arm or a liquid sample washed into the flask via the sidearm with a minimum quantity of deoxygenated deionised water.
- The flask is purged for 2 min at 280 mL/min.
- After turning off the nitrogen flow (at the flow meter), a clean plastic disposable syringe is used to add 20 mL of 6 M HCl through the sidearm to achieve a concentration of 1 M HCl (20 mL × 6 M HCl/(100 mL water + 20 mL 6 M HCl)).
- The magnetic stirrer is started and its speed adjusted to enable moderate stirring of the sample.
- Nitrogen is bubbled through the sample for 30 min at 280 mL/min.
- The contents of both H_2S trapping tubes are quantitatively transferred to a 50 mL volumetric flask (using a funnel to avoid spillage). The contents of the traps should be washed into the flask with 0.5 M NaOH solution. The contents are made up to 50 mL with 0.5 M NaOH, stoppered and shaken thoroughly.
- The main reaction flask containing the acid-leached sample is stoppered and the contents are retained for SEM determination.

C.2.2 Sulfide determination

Calibration standards

A working sulfide standard (~2 mM) should be prepared by pipetting 2 mL of the sulfide stock solution into a 50 mL volumetric flask and making it up to volume with deoxygenated 0.5 M NaOH (made in deoxygenated deionised water).

Quantities (0, 50, 100, 200, 300, 500 µL) of the working sulfide standard (~2 mM) are pipetted into a series of 30 mL polycarbonate vials each containing 10 mL of 0.5 M NaOH. The resulting standards have concentrations in the range 0–0.2 mM. The exact concentrations of the standards should be calculated from the standardisation data.

The working standard and all dilutions should be prepared on a daily basis and stored refrigerated (or in the nitrogen-filled glove box).

Sulfide analyses of standards and samples: General method

Methylene blue reagent (MBR) (1 mL) is added per 10 mL of sample (or standard) and the vessels are allowed to sit for 90 min for the methylene blue colour development. It is important to clean the internal stainless steel barrel of the 5 mL pipette after use to avoid corrosion!

Following colour development (90 min), 1 mL of the sample (or standard) is pipetted into a 30 mL polycarbonate vial containing 9 mL 1 M H_2SO_4.

The solution is analysed at 670 nm using a UV-visible spectrophotometer.

An initial guess of the AVS content of the sample is advisable. Always, as a precautionary measure, the samples should be diluted at least 1 mL/10 mL with 0.5 M NaOH (10-fold dilution). However, it is often necessary (for very smelly sediments) to dilute 50- to 100-fold (0.1–0.5 mL/10 mL).

For some spectrophotometers, the standard curve may become less linear after the fourth standard. If the blue colour of the resulting solution is more intense than those within the linear range of the standards, then a further dilution of the sample with 0.5 M NaOH is required before adding the MBR.

The spectrophotometer is used to measure the absorbance at 670 nm. For many spectrophotometers, it is ideal if the samples have a final absorbance of <0.3 since this is the most linear part of the calibration curve. If the absorbance is >0.35, then the samples may be better re-analysed after further dilution. (Note that dilutions must be made before addition of MBR, not after its reaction has taken place.)

C.2.3 Simultaneously extracted metals (SEM)

The stoppered reaction flask is placed on a tared top-pan balance and made up to 140 mL final volume. After shaking, ~110 mL is transferred from the flask to a labelled 120 mL polycarbonate vial and allowed to settle. After settling (3–4 h), the liquid is decanted off (avoiding shaking) into a labelled 30 mL polycarbonate vial for analysis by ICPAES for Cd, Cu, Fe, Mn, Ni, Pb, and Zn.

C.2.4 Quality control

A blank and spike-recovery sample should be run every day. The blank comprises 100 mL of deoxygenated deionised water and a piece of laboratory paraffin film or a plastic dish.

The recovery is carried out on a sub-sample of sediment analysed in the same batch. A known weight of sediment is added to the reaction flask (as described above) followed by a known volume of sulfide standard (ideally ~2 × AVS-content of the sample). The AVS analysis is carried out as described in the method and the amount of sulfide recovered from this addition is calculated.

Sulfide spikes

For a sample with low AVS (e.g. one which is to be diluted 10-fold), a suitable spike would be 5.0 mL of the working standard.

For a sample with high AVS (e.g. 100–200 mmol/kg; i.e. one that is to be diluted 100-fold), a suitable spike would be 4.0 mL of the sulfide stock solution.

C.2.5 Calculations

Calculation of concentration of sulfide stock solution

$$[S^{2-}] = \frac{(T_b - T_s) \times MS_2O_3^{2-} \times 500}{V_s}$$

$$= (T_b - T_s) \times 12.5/Vs$$

where $[S^{2-}]$ is the sulfide concentration (mm), T_s = volume (mL) of thiosulfate solution used up by the sulfide, T_b = volume (mL) of thiosulfate solution used up by the blank, $MS_2O_3^{2-}$ = molarity of the thiosulfate solution (0.025 M), V_s = volume of sulfide standard used (2.0 mL).

Calculation of acid volatile sulfide (AVS) concentration

Calculate the AVS concentration by a regression analysis of the absorbances of the standard solutions (*y*-axis) against the concentration of standards in µmol/mL. The resulting slope and the *y*-intercept are used to find the AVS concentration.

C.3 Rapid method for acid volatile sulfide (AVS) analysis

The alternative method of AVS analysis, the 'rapid' method, is applicable to sediments having AVS concentrations in the range 0.5–300 mmol/kg (sediment, dry weight) (Simpson, 2001). The limit of determination is ~0.5 mmol/kg. The method uses the direct reaction of 'Cline's reagent' (methylene blue) (Cline, 1969) with small amounts of sediment followed by colorimetric determination of AVS, and it offers fast analysis times without the need for specialised glassware or equipment. A comparison between AVS measured by this method and that measured using a purge-and-trap AVS method shows a linear relationship, although the rapid method underestimates the AVS concentration as measured by the purge-and-trap method. Because sulfide is unstable in the presence of oxygen it is necessary to protect samples from exposure to air. It is recommended that samples be frozen immediately after collection, and that all subsequent manipulations (including thawing) are carried out in a nitrogen or argon atmosphere (glove box or bag).

C.3.1 Materials

- Centrifuge capable of 2500 rpm and housing 50 mL polycarbonate centrifuge tubes.
- Nitrogen-filled glove box for sample handling – desirable but not mandatory.
- Drying oven (110°C).
- Analytical balance capable of measuring to ± 3 mg (i.e. to 0.001 g).
- Spectrophotometer (single or double beam) to measure at 670 nm.
- Laboratory paraffin film (e.g. Parafilm® M).

C.3.2 Reagents

As for the purge-and-trap method (Section C.2, above).

C.3.3 AVS procedure

- The frozen sediment sample is thawed in a nitrogen gas-filled glove box.
- The analysis sample is homogenised in the glove box by mixing with a Teflon® spatula.
- A small square (1.5 cm × 2 cm) of laboratory paraffin film is tared on an analytical balance (accuracy ± 0.001 g).
- The weighed square of laboratory paraffin film is carefully transferred to the glove box, and after a small sample of sediment (0.02–0.10 g dry weight) has been smeared onto its surface it is accurately weighed (3 mg) and immediately transferred to a 50 mL centrifuge tube (in a glove box if possible).
- The process is repeated in triplicate for each sediment sample.
- To each centrifuge tube, 50 mL of deoxygenated deionised water (volumetrically) is added from a wash bottle in the glove box, trying not to disturb the sediment on the laboratory paraffin film too much.
- Methylene blue reagent (MBR) (5 mL) is pipetted into each water-filled tube (using a calibrated 5 mL pipette); the tube is capped and inverted five times to mix.

Caution: MBR is toxic.

- After 5 min, the tube is centrifuged at 2500 rpm for 2 min.
- The tubes are allowed to sit in the dark for 90 min for the methylene blue colour development.

Attention: During this period, care must be taken to avoid significantly disturbing the sediment (i.e. no further shaking) because the MBR adsorbs to sediment particles.

C.3.4 Sulfide determination

Sulfide standards

A working sulfide standard (~2 mM) should be prepared by diluting a 50 mM sulfide stock solution into a 50 mL volumetric flask and making it up to volume with deoxygenated 0.5 M NaOH (made in deoxygenated deionised water).

The working standard and all dilutions should be prepared on a daily basis and stored refrigerated (or in the nitrogen-filled glove box).

Quantities (0, 50, 100, 200, 300, 500 µL) of working sulfide standard are pipetted into a series of 30 mL polycarbonate vials each containing 10 mL 0.5 M NaOH. The resulting standards have concentrations in the range 0–0.2 mm. The exact concentrations of the standards should be calculated from the standardisation data.

Sulfide analyses of standards and samples

Following colour development of standards and samples, dilution is achieved by pipetting 1 mL of the standard or sample into a 30 mL polycarbonate vial containing 9 mL of 1 M H_2SO_4.

The solution absorbance is measured at 670 nm using a UV-visible spectrophotometer.

For this method, if the absorbance of the sample is greater than that of the standard with the highest sulfide concentration, then the sample should be re-analysed using a smaller amount of sediment (per unit volume). This is in preference to further dilution of the sample.

C.3.5 Calculating the AVS concentration

The AVS concentration is calculated by regression analysis of the absorbances of the standard solutions (y-axis) against the concentration of standards in µmol/L. The resulting slope and the y-intercept are used to calculate the AVS concentration in the sample. The AVS concentrations should be reported on a dry-weight basis following determination of the water content of the sample.

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Appendix D: Protocol for whole-sediment bioassay using the marine microalga *Entomoneis* cf *punctulata*

Merrin S. Adams

D.1 Introduction

This appendix describes a protocol for testing the short-term adverse effects of contaminated sediment on the marine unicellular microalga *Entomoneis* cf *punctulata* (Fig. D.1) after static 24-h exposures. This method is based on the protocol of Adams and Stauber (2004) and is applicable to sediments containing toxicants, either individual or as mixtures. Toxicity identification evaluation (TIE) procedures (Phase I) for hydrocarbon-contaminated sediments are also available for this test (Simpson *et al*., 2007).

Microorganisms are vital in nutrient cycling and the degradation of organic matter in sediments. Even though benthic microorganisms do not ingest sediment particles, they are in intimate contact with sediments and associated pore waters. Benthic algae (microphytobenthos) play an important role in stabilising sediments, providing habitat, modulating chemical transformations and remobilising metals at the sediment–water interface. They are a common food source for invertebrates (e.g. amphipods) and hence have the potential to facilitate transfer of sediment contaminants to higher trophic levels.

Short-duration tests that detect acute sub-lethal endpoints, such as inhibition of enzyme activity, show promise for overcoming some of the limitations of standard algal growth tests. Enzyme inhibition, rather than inhibition of algal growth, has been found to be a more suitable endpoint for algal whole-sediment tests because toxic effects are not masked by stimulation from ammonia in sediments. The need for accurate quantification of microalgae in sediments has also hampered the ability to carry out growth rate inhibition bioassays in sediment.

Inhibition of esterase activity in algae has been shown to relate well to metabolic activity (Gala and Giesy, 1990) as an indicator of cell health, and it can be measured readily via fluorescence. (Experiments measuring esterase activity in *E.* cf *puntulata* have, however, shown that algal cells with inhibited esterase activity can still grow and divide, so esterase activity is not necessarily an indicator of cell viability as has been suggested in the literature.)

D.2 Method basis and summary

This toxicity test measures the acute (sub-lethal) toxicity of contaminants in estuarine or marine sediments to a microalga following a 24-h exposure. The test is a cellular enzyme

activity bioassay that measures the inhibition of esterase activity in the benthic alga *E.* cf *punctulata* after exposure to contaminated sediment compared to the same measure in a control sediment.

Esterases are a group of enzymes involved in phospholipid turnover in cell membranes. The esterase activity in algae is decreased by toxicants, and this can be measured by flow cytometry using the non-fluorescent stain fluorescein diacetate (FDA). Healthy cells take up the lipophilic stain by diffusion across the cell membrane to the cytoplasm where enzymes cleave the diacetate ester bonds to yield fluorescein, a hydrophilic anion that is retained by intact viable cells. Fluorescein fluoresces in the green region of the visible light spectrum when excited by blue light. The presence of toxicants can be observed as a decrease in intracellular fluorescein fluorescence intensity, which is measured by flow cytometry.

Flow cytometry is a rapid method for the measurement of the light-scattering and fluorescence properties of individual cells in a moving fluid. Microalgae are ideal for flow cytometric analysis because they are single-celled organisms containing photosynthetic pigments, such as chlorophyll-*a*, which autofluoresce when excited by blue light (488 nm) and are thus easily distinguishable from sediment particles. Fluorescein fluorescence can be detected by flow cytometry and reflects both esterase activity and cell membrane integrity, indicating cell health (Dorsey *et al.*, 1989; Franklin *et al.*, 2001). Two light-scatter detectors provide information on the morphology of the cell. The forward-angle light scatter (FSC, <15°) detector provides information on cell size, while the side-angle light scatter (SSC, 90°) provides information on internal complexity and granularity. Fluorescence is detected simultaneously at a range of wavelengths by photomultiplier tubes with different fluorescence emission filters (e.g. FL1, 515–545 nm, green fluorescence; FL2, 564–606 nm, orange fluorescence; FL3, >650 nm, red fluorescence).

In this protocol, the use of a BD-FACSCalibur™ (Becton Dickinson BioScience, San Jose, CA, USA) flow cytometer to measure algal esterase activity is described. It should be noted that instrument settings and methods of analysis may vary between flow cytometers. The flow cytometric toxicity tests require considerable investment in equipment and skilled operators, but once the individual test protocol is established, it is relatively easy to train operators in its routine use.

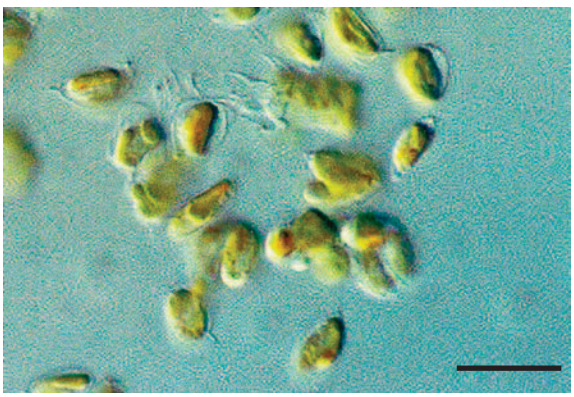

Figure D.1. Live cells of the microalga *Entomoneis* cf *punctulata,* imaged by differential interference contrast microscopy (Normaski) (courtesy of Dr Richard Knuckey). Scale bar: 10 µm.

D.3 Method

D.3.1 Equipment

- Flow cytometer (e.g. FACSCalibur, Beckman Dickinson, or equivalent).
- Constant temperature chamber or temperature controlled room with 12-h light (toxicity test: 1–10 µmol photons/m^2/s; culture: 50–70 µmol photons/m^2/s) : 12-h dark cycle, maintained at 21 ± 1°C.
- Test vials: 20 mL glass scintillation vials or 30 mL polycarbonate vials.
- Algal culture flasks: 250 mL Schott bottles or flasks with glass lids.
- Dissolved oxygen, pH and salinity meter.
- Top-pan balance (0.01 g) and analytical (0.0001 g) balance.
- Uncontaminated seawater (35‰, filtered to 0.45 µm and stored at 4°C).
- Fluorescein diacetate (FDA), Sigma.
- Copper sulfate ($CuSO_4 \cdot 5H_2O$), AR grade.

D.3.2 Seawater

Seawater, used for culturing and as overlying water in toxicity tests, is collected from an uncontaminated site and filtered to 0.45 µm. The salinity of filtered seawater is typically 33–35‰ and it can be adjusted to a lower salinity (as required) by the addition of deionised water (for example, Milli-Q® water) if necessary.

D.3.3 Control sediment

Sediments used as the control treatment in toxicity tests are collected from uncontaminated sites with a range of grain size distributions (fine and coarse sediments). In each test, the grain size of the control sediment should be matched as closely as possible to the grain size of the test sediment. Additional control sediments with grain sizes similar to those of the test sediment can be prepared by mixing an appropriate weight of two sediments (fine and coarse). Control sediment is collected using a stainless-steel spade or hand trowel and is press-sieved (wearing gloves) through a 2 mm stainless-steel mesh sieve on-site to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in portable cooler boxes with ice or cooler bricks and returned to the laboratory and stored at 4°C for a maximum of 2 months. Sediment is equilibrated to the temperature of the test before being used. As the test measures algal enzyme activity in test sediments compared to that in control sediments, it is crucial that the control sediment not only has low concentrations of contaminant but also has physico-chemical properties, such as grain size and pore-water salinity, that are similar to those of the test sediment.

D.3.4 Test sediment

Test sediments should be collected and stored in containers made of inert materials to prevent contamination. The sediments should be chilled when collected, transported cold and stored in the dark at 4°C. The sediments should not be frozen nor allowed to dry out and should be used in tests as soon as possible. Before use, test sediments must be allowed to equilibrate to the test temperature. The particle size distribution of the sediments should be measured before they are used so that a suitable control sediment can be selected. Other sediment characterisation should include measurements of the salinity and pH of pore water, and of the pore-water concentrations of metals and/or organic contaminants (based on known or expected contaminants in the sediment samples) and total organic carbon.

D.3.5 Test organisms

The unicellular benthic marine alga *Entomoneis* sp. (strain no. CS-426) can be obtained from the CSIRO Australian National Algae Culture Collection (Hobart, Australia). It was originally isolated from Little Swanport (42°20':147°56'), on the east coast of Tasmania by Richard Knuckey in 1995. The isolate was identified as *Entomoneis* cf *punctulata* Osada et Kobayashi (CS-426) by Dr Gustaaf Hallegraeff (University of Tasmania, Australia).

Culturing of algae

Algae are cultured in a modified half-strength f Medium (Guillard and Ryther, 1962) with the iron and trace element concentrations halved (Table D.1). Cultures are maintained in 50 mL of sterilised (autoclaved) medium in 250 mL sterilised Schott bottles. Each week, 1 mL of 1-week old algae is transferred under axenic conditions to fresh medium prepared 1–3 days in advance. Cultures are maintained on a 12-h light : 12-h dark cycle (Philips TL 40 W fluorescent daylight, 50–70 μmol/m^2/s) at 21°C.

Preparation of algae for toxicity testing

Cells in exponential growth (5 days old) are used in the algal bioassay after being washed three times with filtered seawater to remove algal culture medium (centrifuged at 700 g for 7 min each wash). Algal cells are inoculated into the test vials immediately after the cells have been washed (i.e. test treatments are prepared before preparing the algal inoculum).

D.3.6 Test protocol

A summary of the test protocol is shown in Table D.2. Sediments are tested in quadruplicate at one test concentration (10% w/v), together with a control sediment of a similar grain size. A 1 g sample of wet sediment is weighed into a glass scintillation vial (or a 30 mL polycarbonate vial) and distributed to evenly cover the base of the test vial. Seawater (9 mL) is then gently added to the vial with minimal disturbance to the sediment. A replicate is prepared for pH measurements throughout the test. Each vial is carefully inoculated just above the sediment surface with 5–9 × 10^4 cells/mL of a pre-washed algal suspension. Samples are incubated at 21°C on a 12-h light : 12-h dark cycle at 1–10 μmol photons/s/m^2 without disturbance for 24 h.

Analysis of algal esterase activity

Analysis of the esterase activity in the treatments prepared for quality assurance (QA) is carried out before analysing the test sediments to ensure that the QA criteria for the toxicity test are met.

After a 24-h exposure, each vial is shaken briefly to resuspend the algae and left for 30 s to allow large sediment particles to settle. A 5 mL sub-sample of the supernatant is homogenised in a hand-held tissue grinder. Fluorescein diacetate (FDA) (63 μL of 1 mM stock in acetone, prepared daily) is added to a 2.44 mL homogenised sub-sample and incubated for 5 min. Immediately before analysis, a Microtox® solid-phase filter column (~50 μm pore size), pre-rinsed with seawater, is used to filter a small fraction of the sample to remove any remaining large sediment particles that could potentially block the flow cytometer aperture.

Samples are analysed for esterase activity (measured as fluorescein fluorescence) with excitation at 488 nm, and the resulting fluorescence is collected in detector FL1 (i.e. green fluorescence) using the FACSCalibur flow cytometer. The instrument flow rate is set on high (≈60 μL/min). Sediment particles and unhealthy cells are excluded from the analysis by setting a threshold on chlorophyll-*a* (red) fluorescence, to the left of the algal population. The algal population is identified using a plot of chlorophyll fluorescence (FL3) versus side scatter (SSC).

Table D.1. Culture medium (f Medium)[a] for *Entomoneis* cf *punctulata*

Stock solution no.	Nutrient	Stock solution		Volume of stock solution per 50 mL of seawater (mL)
		Mass added	Stock volume	
1	$NaNO_3$	7.5 g	100 mL	0.100
2	$Na_2SiO_3.5H_2O$	6.25 g	250 mL	0.050
3	Iron(III) citrate[b] ($C_6H_5O_7Fe.5H_2O$) Citric acid	0.45 g 0.45 g	100 mL	0.050
4	**METALS**[c]		1 L	0.050
	$CoCl_2.6H_2O$	10 mg		
	$CuSO_4.5H_2O$	9 mg		
	$Na_2SiO_3.5H_2O$	7 mg		
	$MnCl_2.4H_2O$	180 mg		
	$ZnSO_4.7H_2O$	22 mg		
5	**VITAMINS**[d]			0.050
	Cyanocobalamin (B12) ($C_{63}H_{88}N_{14}PCo$)	0.025 g	250 mL	
	Biotin (H) ($C_{10}H_{16}N_2O_3S$)	0.05 g	500 mL	
	Thiamine (B1.HCl) ($C_{12}H_{17}N_4OSCl.HCl$)	0.05 g	250 mL	
6	$Na_2MoO_4.2H_2O$	12.6 mg	1 L	0.050
7[e]	$NaH_2PO_4.2H_2O$	2.5 g	250 mL	0.050

[a] f Medium (Guillard and Ryther, 1962).
[b] Iron(III) citrate and citric acid, 0.45 g of each in 100 mL; may need to be warmed to aid solubility.
[c] Metal concentrations in this medium are half the concentration of the original f Medium.
[d] Add 2.5 mL of vitamin B12 stock and 2.5 mL of biotin to a 250 mL volumetric flask containing 0.05 g thiamine. Make up to volume with deionised water.
[e] Stock solution no. 7 is sterilised independently of the f Medium and added immediately before transferring algae.

A secondary threshold on SSC may be required for samples with a high sediment particle count. The fluorescein fluorescence of only the algal cells is plotted as a histogram of FL1 (green) fluorescence versus cell count. The region S2 is manually defined around the FL1 fluorescence intensity of the sediment control. The percentage of cells falling into regions S1 (decreased FL1 fluorescence), S2 (normal FDA fluorescence) and S3 (enhanced FDA fluorescence) are recorded and expressed as a percentage decrease in S2/S3 compared to sediment controls (Fig. D.2). The pH of each sample is measured at the end of the test.

D.3.7 Quality assurance

Negative control

Seawater negative controls are included in each toxicity test (10 mL of seawater only, in triplicate) to measure the fluorescein fluorescence, and hence esterase activity, associated with healthy (untreated) algal cells. An additional vial is prepared for measuring pH at the beginning and end of the test. Each vial is inoculated with algae (2–4 × 10^4 cells/mL) and

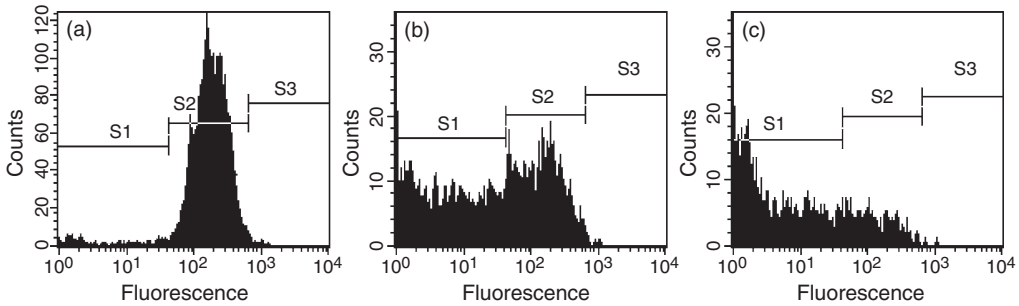

Figure D.2. Flow cytometric histogram showing shifts in esterase activity (FL1 fluorescence versus cell number) of *Entomoneis* cf *punctulata* after a 24-h exposure to copper in seawater. (a) 0 µg Cu/L (Control), 4% in S1; (b) 85 µg Cu/L, 58% in S1; (c) 250 µg Cu/L, 78% in S1. Exposure shown is in seawater only (no sediment).

Table D.2. Summary of toxicity test conditions for the *Entomoneis* cf *punctulata* esterase inhibition test for sediments

Test type	Static
Temperature	21 ± 1°C
Light quality	Daylight or cool-white fluorescent lighting
Light intensity	1–10 µmol photons/s/m^2
Photoperiod	12-h light: 12-h dark
Test chamber	20 mL glass vial or 30 mL polycarbonate vial with screw-cap lids
Test solution volume	10% w/v (1 g sediment plus 9 mL of seawater)
Renewal of test solutions	None
Age of test organisms	5 days
Growth phase	Exponential
Initial cell density	5–9 × 10^4 cells/mL
No. of replicate chambers/sample	4
Dilution water	Seawater (filtered to 0.45 µm)
Acceptable pH range	6.5–8.5
Acceptable salinity range	15–35‰
Test duration	24 h
Test endpoint	Esterase activity
Test acceptability	Distinct separation between fluorescence intensity of negative and positive controls; >80% healthy esterase activity in sediment controls within 4 replicates; Reference toxicant EC50 within Cusum chart limit

incubated under the same conditions as described for the sediment bioassay. Seawater controls are analysed using the procedure outlined below.

Positive control

One additional seawater control (10 mL of seawater) is prepared and inoculated with algae for use as a positive control. When the test is initiated (t_0), 0.4 mL of formalin is added to the

vial (that is, 4% formalin) and the vial is stored at 4°C for 24 h. In this treatment, algal cells still show strong chlorophyll fluorescence while esterase activity is significantly inhibited.

Reference toxicant

A reference toxicant, copper, is tested in parallel with each toxicity test to assess the relative sensitivity of the algae used in the toxicity test and the precision and reliability of the data produced under standardised test conditions. A water-only exposure is recommended for reference toxicants. Copper, as copper(II) sulfate, is added to 10 mL of seawater to give concentrations of 25, 50, 100 and 300 µg Cu/L, in duplicate. Two additional vials are prepared for measuring (i) copper concentrations, and (ii) pH at the beginning and end of the test. Each vial is inoculated with algae ($2–4 \times 10^4$ cells/mL) and incubated under the same conditions as described for the sediment bioassay. Seawater controls are analysed following the procedure outlined below. Samples for copper analysis are filtered through an acid-washed 0.45 µm membrane syringe filter and acidified to 2% nitric acid (high purity grade) for analysis by inductively coupled plasma atomic emission spectrometry (ICPAES).

A definitive test consisting of at least 5 concentrations, in quadruplicate, should be carried out regularly, and a cumulative summation (Cusum) chart of EC50 values generated, with EC50 values within 2 standard deviations of the mean indicating acceptable test criteria.

D.3.8 Analysis of algal esterase activity

After a 24-h exposure, each vial is shaken and a sub-sample (2.44 mL) is transferred to a clean glass vial. After incubation with FDA (63 µL of a 1 mM FDA solution in acetone, prepared daily) for 5 min, algal esterase activity is measured by flow cytometry using a high flow rate (60 µL/min). Unhealthy cells are excluded from the analysis by setting a threshold on chlorophyll-*a* fluorescence (FL3, >650 nm). The algal population is identified using a plot of chlorophyll fluorescence (FL3) versus side scatter (SSC), as described above. The pH of each sample is also measured at the end of the test.

Criteria for test acceptability

The use of negative controls (untreated algal cells with healthy esterase activity) and positive controls (cells with inactivated esterase activity) defines the expected FL1 fluorescence intensity for the 'no effect' test concentrations and '100% effect' (inhibition) in esterase activity, respectively (Fig. D.3). A separation of <10% overlap in FL1 fluorescence intensity is required to ensure that shifts in FL1 fluorescence can be quantified. Control FDA-stained cells should display a normal distribution for the algal population on the FL1 histogram. The control region, S2, can then be confidently defined around >90% of the cells.

The EC50 value for the reference toxicant should be within two standard deviations of the Cusum chart of EC50 values.

D.3.9 Data analysis

Each replicate is expressed as a percentage decrease in S2/S3 (healthy FDA fluorescence intensity) compared to sediment controls according to the following equation:

$$(100 - \%S1_t) \div (100 - \%S1_c) \times 100$$

where $\%S1_t$ is the percentage of treated cells in S1 and $\%S1_c$ is the average percentage of control (untreated cells) in S1.

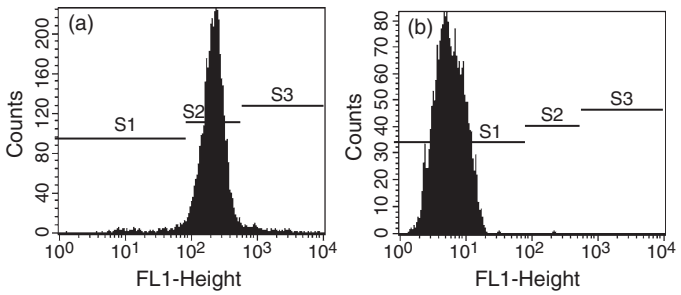

Figure D.3. Flow cytometric analysis of (a) negative control cells (healthy cells); (b) positive control cells (formalin-fixed cells) after incubation with FDA. Histograms show FL1 (fluorescein, green) fluorescence intensity versus cell number.

A sediment sample is defined as toxic if there is more than a 20% inhibition in esterase activity compared to the control sediment (that is, its esterase activity is <80% of the control), and if its esterase activity is significantly less than the esterase activity in the control sediment ($P \leq 0.05$). This criterion is based on test variability during the test development period and is in agreement with published protocols for amphipod acute toxicity tests using whole sediment (Environment Canada, 1997). For use in a toxicity line of evidence as part of a weight of evidence assessment (see Chapter 1 Table 1.1), the toxicity data are assessed as non-toxic if the response is <20% inhibition in esterase activity compared to a control response, toxic with significant effects for 20–50%, and toxic with significant and major effects if the response is ≥50%.

The proportional data are arcsin transformed and tested for normality of distribution (Shapiro-Wilk test) and homogeneity of variance (Bartlett's test) before hypothesis testing. If the assumptions of normality and homogeneity are met, the Dunnett's test can be used; if these assumptions are not met, Steel's test can be used. Student's *t*-test may also be used to test for significant differences between two treatments.

For toxicity tests where a concentration–response relationship is observed (e.g. QA reference toxicant test), multiple treatments are compared to a single control. The Trimmed Spearman-Karber method is then used to determine the EC50 value (the concentration of test sample to cause a 50% inhibition in esterase activity). Probit analysis may also be applied to determine ECx values.

D.4 Further information and future development of algal sediment toxicity tests

D.4.1 Sensitivity of *E.* cf *punctulata* esterase inhibition toxicity test to contaminants

In general, sediment toxicity to microalgae has been shown to be related to water routes of exposure within sediments compared to direct contact with sediment-bound contaminants (Adams and Stauber, 2004). Esterase activity in *E.* cf *punctulata* is, in general, more sensitive to organic than metal contaminants in sediments. Studies with copper-spiked sediment have shown that a sediment concentration of 2500 mg Cu/kg (with a pore-water dissolved copper concentration below that known to cause toxicity to the alga) is not toxic to esterase activity in *E.* cf *punctulata* (M. Adams, unpublished results). In contrast,

concentration–response relationships are observed for hydrocarbon-contaminated sediment with effects observed at 60 mg PAHs/kg (normalised to 1% total organic carbon) (Simpson *et al*., 2007).

The tolerance of *E.* cf *punctulata* esterase activity to ammonia and sulfide, two common natural stressors in sediments, is described in Adams and Stauber (2004). *Entomoneis* cf *punctulata* is tolerant to 8.5 mg/L ammonia (as total ammonia at pH 8.1 ± 0.1) and 2 mg/L sulfide (as S^{2-}).

D.4.2 Toxicity identification evaluation (TIE)

A TIE method for identifying hydrocarbon-contaminated sediment has also been developed for the esterase algal-sediment bioassay. Addition of powdered coconut charcoal to hydrocarbon-contaminated sediment decreases the toxicity of hydrocarbon-contaminated sediments to *E.* cf *punctulata*. These test methods are described in more detail by Simpson *et al.* (2007).

D.4.3 Toxicity of waters and effluents to *E.* cf *punctulata*

The toxicity of water samples, such as effluents, leachates and estuarine and marine waters, to *E.* cf *punctulata* can also be assessed using the esterase inhibition bioassay by following the procedures outlined above.

Chronic growth-inhibition bioassays can also be utilised to assess the chronic toxicity of waters or effluents to the alga using methods described for other marine microalgae such as *Ceratoneis closterium* (previously *Cylindrotheca closterium* and *Nitzschia closterium*) (Stauber *et al*., 1994; Franklin *et al*., 2005). *Entomoneis* cf *punctulata* maintains exponential growth in a minimal nutrient seawater toxicity test medium (seawater supplemented with nitrate and phosphate) for up to 48 h (M. Adams unpublished). Therefore, growth rate inhibition tests with *E.* cf *punctulata* are carried out over 48 h as opposed to the 72-h test durations generally adopted for other microalgal species.

D.4.4 Chronic sediment toxicity tests with benthic microalgae

Recent developments in algal toxicity testing of sediments have focused on chronic toxicity to microalgae measured as an inhibition in growth rate. The need for accurate measurements of algal cell density in sediments has limited the development and application of growth-inhibition bioassays because it is not possible to quantify algal cell density in sediment samples. Toxicity tests measuring the inhibition in growth rate of the microalga *Ceratoneis closterium* (previously *Cylindrotheca closterium*) have been developed; they measure algal cell density using fluorescence microscopy (Moreno-Garrido *et al*., 2003). However, toxicity tests using this technique have been limited to sediments with a fine fraction (<63 μm) no greater than 15%.

Our laboratory has developed a method for using chlorophyll measurements as a surrogate for cell density (Strom, 2011). Throughout the 10-day duration of the test, chlorophyll concentrations are measured spectrophotometrically after an acetone extraction of the sediment. This technique holds promise for use as a chronic toxicity test for sediments.

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Appendix E: Protocol for 10-day whole-sediment sub-lethal (reproduction) and acute toxicity tests using the epibenthic amphipod *Melita plumulosa*

David A. Spadaro and Stuart L. Simpson

E.1 Introduction

This appendix describes methods for testing the sub-lethal (chronic) adverse effects of potentially contaminated sediment on the survival and reproduction of the epibenthic detritivorous amphipod species *Melita plumulosa* (Amphipoda, Gammaridea, Melitidae) (Fig. E.1). This species is native to estuaries in eastern Australia. The method represents a sensitive and robust approach for undertaking whole-sediment toxicity tests with acute lethality and sub-lethal chronic test endpoints.

Melita plumulosa is a free-ranging scavenger that obtains food by ingesting detritus and fine sediments and this causes dietary exposure to sediment-bound contaminants (King *et al*., 2005; Simpson and King, 2005; Strom *et al*., 2011; Campana *et al*., 2012).

Based on the method of Mann *et al.* (2009), the test measures lethality and the reproduction of *M. plumulosa* following exposure to undiluted test sediments over a 10-day period. The species is suitable for testing for the effects of contaminants in a full spectrum of sediment types (sand to silt, high to low organic carbon) (Spadaro *et al*., 2008; Simpson and Spadaro, 2011). The 10-day reproduction endpoint for *M. plumulosa* is both sensitive (i.e. responds well when bioavailable contaminant concentrations exceed guidelines) and robust, making it ideal for sediment quality assessments (Mann *et al*., 2010; Simpson and Spadaro, 2011; Simpson *et al*., 2013).

Melita plumulosa was selected as an excellent species for conducting whole-sediment toxicity tests, after five benthic amphipod species collected from estuaries near Sydney (Australia) were screened for their sensitivities to metal-contaminated sediments (King *et al*., 2006). Additional factors relate to its ease of culture, tolerance to a range of salinities (5–35‰) and sediment types (silt to sand) (Hyne *et al*., 2005; Simpson and Spadaro, 2011), and exposure to sediment through ingestion of fine particles (King *et al*., 2005; Strom *et al*., 2011; Campana *et al*., 2012).

Melita plumulosa is found in silty to silty-sand sediments in temperate (e.g. 12–28°C) and generally estuarine locations (e.g. 1–34‰). *Melita plumulosa* is epibenthic and, while it

Figure E.1. Adult male amphipod *Melita plumulosa* (10 mm in photo; 7–12 mm range for males and 5–9 mm range for females).

does not construct permanent burrows, it does burrow to up to 5 mm depth. The species deposit-feeds on sediment and detritus. In water-only exposures without feeding, juvenile and adult *M. plumulosa* will typically survive for 4 or 10 days respectively, but will not reproduce without an adequate sediment substrate (Hyne *et al*., 2005; Spadaro *et al*., 2008).

The full life-cycle of *M. plumulosa* (juvenile to juvenile) extends for 30–40 days, and the species may live for up to 12 months (Hyne *et al*., 2005). The female fertility is greatest when they are cultured in silty sediments at 22–25°C and 25–32‰ salinity (Hyne *et al*., 2005; Spadaro *et al*., 2008). Fertility drops markedly when temperatures are less than 20°C, or waters have 5‰ > salinity > 36‰, or when sediments contain <20% silt (Hyne *et al*., 2005; Spadaro *et al*., 2008). However, by feeding with commercially available fish food (e.g. Sera Micron, 1 mg per adult amphipod twice per week), survival and acceptable reproduction rates can be achieved over 10 days in 100% sand substrates. The survival and reproduction of *M. plumulosa* are not significantly affected by light illumination conditions, and natural day–night conditions in the laboratory are suitable for sustained culturing, and a 12-h light : 12-h dark photoperiod for testing (Spadaro *et al*., 2008; Simpson and Spadaro, 2011).

Amphipods are an abundant and important component of the soft-bottom estuarine and marine benthic community. They feed principally on microbial and algal species. Many epibenthic species, including *M. plumulosa*, directly ingest sediment particles and are therefore directly exposed to sediment-bound contaminants. Amphipods are a principal prey item of many birds, fish and larger invertebrates and hence have the potential for trophic transfer of contaminants from sediments to higher trophic levels. The ecological importance of amphipods, their wide distribution and high abundance, their ease of handling and ability to be cultured in the laboratory, and their sensitivity to contaminated sediments make them appropriate species for sediment toxicity testing. Amphipods have frequently been used as test organisms in whole-sediment toxicity tests because they meet many of the criteria used for selecting test organisms (ASTM, 2014; Castro *et al*., 2006; Scarlett *et al*., 2007; Greenstein *et al*., 2008).

The test procedure describes a rapid standardised lethal and sub-lethal toxicity test that provides an indication of the potential toxicity of estuarine and marine sediment samples

to local biota. The reproduction endpoint provides information that indicates potential for long-term effects at the individual and population levels.

E.2 Method basis and summary

Full life-cycle tests are usually defined as those that expose animals from one generation of eggs–embryos–juveniles, through the various life stages to reproducing adults and to the next eggs–embryos–juveniles. In some cases, the exposure of animals from hatching to reproductive maturity is also classified as a life-cycle test. Such life-cycle tests allow assessment of adverse effects of contaminant exposure on organism development and reproduction.

Challenges associated with conducting life-cycle tests often include the long life-cycles of many species, which may increase the variability arising from non-contaminant factors (e.g. sediment properties), the effects of confounding factors such as the source of food addition, and the cost of the tests compared with acute tests.

The life-cycle of *M. plumulosa* from juvenile to reproductively mature adult is completed within four to six weeks (Hyne *et al.*, 2005). Due to cost and time constraints, performing a full life-cycle test using this species is not feasible for most assessments (Gale *et al.*, 2006). However, as is the case with many crustaceans, fecundity (the number of offspring produced by an individual) has been demonstrated to be a significantly more sensitive endpoint for this species than survival or growth (Gale *et al.*, 2006; Spadaro *et al.*, 2008). With the aim of substantially shortening the test, Mann and Hyne (2008) undertook a detailed study of the reproductive stages of embryological development in *M. plumulosa* in order to rapidly assess reproductive toxicity. That study formed the basis for developing what was initially a 13-day amphipod reproduction test that encompasses gametogenesis, fertilisation, and embryo development before hatching (Mann *et al.*, 2009).

The 10-day amphipod reproduction bioassay described here was adapted from Mann *et al.* (2009) and measures, along with adult survival and reproduction, the number of embryos and <1 day-old juveniles in the second brood following exposure of *M. plumulosa* to test sediments over a 10-day period. The long test durations used in many chronic sediment tests frequently result in relatively great variability in the performance of those tests (Greenstein *et al.*, 2008; Kennedy *et al.*, 2009). However, an adequately small level of variability is achieved with this rapid amphipod reproduction bioassay (Simpson and Spadaro, 2011). For uncontaminated sediments, the variability in reproduction is largely unaffected by differences in sediment particle size (0–100% silt) and total organic carbon (0–8% TOC). The test provides similar sensitivity to a full life-cycle test, but with less variability in controls, making it more convenient for use in routine toxicity assessments.

Sediments are homogenised immediately before being added to test vials (40 g whole sediment per 250 mL beaker). A 200 mL volume of filtered seawater (<1 µm, 30‰ salinity) is added and each beaker is incubated at 21°C with aeration overnight to allow sediments to settle. Four to five replicates are used per sediment. The following day, 180 mL of overlying water is siphoned off and replaced with new seawater with care to minimise sediment resuspension. Six gravid females (gravid for <24 h) and six males (isolated from laboratory cultures) are randomly assigned to each beaker. Treatments are fed at a rate of 0.25 mg of Sera Micron fish food/amphipod twice a week. The sediments are renewed after 5 days by gently sieving away the adults and placing them into the same fresh sediment, which has

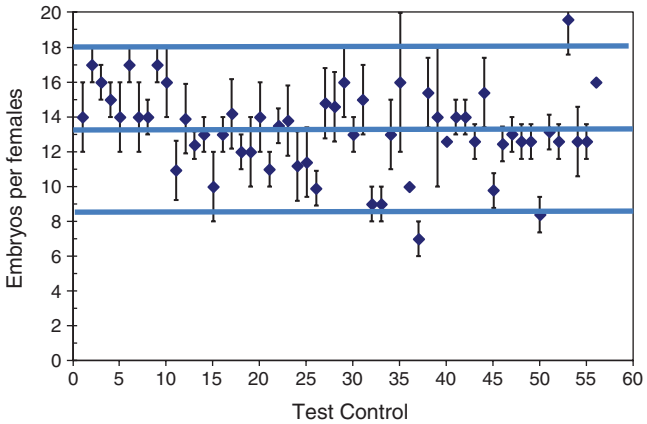

Figure E.2. Control chart of acceptable reproduction, measured in 57 control treatments (solid horizontal line = expected mean number of embryos per female; dashed lines show 2 standard deviations).

been equilibrated overnight, thus allowing for the removal of juveniles from the first brood. The first brood is typically unaffected by contaminants in the test sediment because they were already 'conceived' before exposure to test sediments. On day 10, the females are carefully removed by sieving (600 µm mesh) and the number of embryos per female is counted by microscopy. The sediment is also checked for juvenile amphipods that have escaped the marsupium during the latter stages of the test, by sieving the sediment through 180 µm mesh. The total numbers of embryos and <1 day-old juveniles are summed and expressed as a percentage of the control. For quality assurance purposes, 8 to 19 juveniles per female are required in all controls for tests to be considered acceptable (Fig. E.2). When controls fail this criterion, all tests in the batch (i.e. controls and test sediments) are repeated with a new population of amphipods, because unknown factors may be confounding the test outcomes.

E.3 Method

E.3.1 Equipment

- Constant temperature cabinet or temperature-controlled room with 12-h light (3.5 µmol photons/s/m^2) : 12-h dark cycle, maintained at 21 ± 1°C.
- Test chambers: 250 mL glass beakers.
- Acrylic beaker lids.
- Air supply and tubing.
- Diluent/control filtered seawater; 0.45 µm.
- Particle size matched control sediment.
- Dissolved oxygen, pH and salinity/conductivity meters.
- Sieves: stainless-steel 600 µm mesh and 180 µm mesh; 2 mm plastic mesh.
- Plastic weighing boats.
- Plastic pipette (wide bore).
- Stainless-steel spade or hand trowel to collect control sediment.
- Portable cooler box with ice or cooler blocks.

E.3.2 Seawater

Seawater used for culturing and acclimatising amphipods, and as overlying water in toxicity tests, is collected from an uncontaminated site and is filtered to 0.45 µm. The salinity of filtered seawater is typically 33–35‰ and is adjusted to a lower salinity (as required) by the addition of deionised water.

E.3.3 Control sediment

Uncontaminated silty sediments from estuarine locations are ideal for culturing the amphipod *M. plumulosa,* and for potential use in controls in toxicity tests. Suitable sites should have >95% fine particles (<63 µm) and low concentrations of metal and organic contaminants.

Sediments for culturing and controls can be collected using a stainless-steel spade or hand trowel and are press-sieved (wearing gloves) through a 2 mm plastic mesh sieve on site to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in cooler boxes with ice or cooler blocks and returned to the laboratory and stored at 4°C for a maximum of 2 months. Sediments are placed at room temperature (21 ± 2°C) for a minimum of 24 h to allow them to equilibrate to the test and culturing temperature. As survival in test sediments is compared to survival in control sediments in the toxicity test, it is crucial that the control sediment not only has low contaminant levels but also has similar physico-chemical parameters to the test sediment, including grain size and pore-water salinity. Uncontaminated sediments with a variety of properties can be collected from different estuarine locations. For control sediments, greater variation in physico-chemical properties can be achieved by mixing silty and sandy sediments with clean sand.

E.3.4 Test sediment

Test sediments should be collected and stored in containers made of inert materials to prevent contamination. Sediment should be chilled when collected, shipped on ice and stored in the dark in a refrigerator at 4°C. It should not be frozen or allowed to dry out and should be used in tests as soon as possible and within 8 weeks of receipt. Prior to its use, test sediment is placed at room temperature (21 ± 2°C) for a minimum of 24 h to allow it to equilibrate to the test temperature.

E.3.5 Test organisms

Collection, handling and culturing of amphipods

Amphipods used in tests are obtained from laboratory-maintained cultures. Stock cultures may be established from animals collected from inter-tidal mud flats at Brooklyn in the Hawkesbury River, north of Sydney, New South Wales, Australia. The species is widely distributed and should readily be found in similar locations elsewhere in south-eastern Australia. Amphipods are collected at low tide from the top few millimetres of sediment or from the under-surface of rocks and shell grit. They are placed into clean plastic containers with water and a thin layer of sediment collected at the same time. Containers with amphipods are transported back to the laboratory in portable coolers maintained at ambient temperature or lower. Amphipods are kept in a temperature-controlled laboratory at 21 ± 2°C in clean plastic holding trays (40 cm × 30 cm × 10 cm) containing 2 mm-sieved sediment to a depth of 1–2 cm and ~5 cm of overlying water (0.45 µm filtered seawater at a salinity of 30‰). Typically a maximum of 600 adults may be kept per tray without overcrowding or any reduction in reproduction and growth. Overlying water in trays is continuously gently aerated and trays are covered with foil to minimise light disturbances to the amphipods.

During culturing, amphipods are fed 1 mg/adult of Sera Micron fish food in combination with 1×10^5 cells of the alga *Phaeodactylum tricornutum* per culture, twice weekly. The overlying water in holding trays is renewed every 2 weeks (by gentle siphoning) and sediment is changed every 6–8 weeks. At the sediment changes, amphipods are sieved from sediments and sorted into age/size classes before being put into separate holding trays with fresh sediment and water. This ensures that amphipods of a uniform and known size and age are used for the toxicity tests.

Prior to the start of the test, amphipods are slowly acclimatised to the test salinity as required (if test salinity is different from 30‰), by increasing or decreasing the salinity of the overlying water in aquaria by 1–2‰ per day. At least three-quarters of the water is removed and replaced with filtered seawater at the appropriate salinity (prepared by the addition of deionised water (for example, Milli-Q® water) to natural filtered seawater at 35‰) each day. Amphipods are fed (as above) after each water change.

Individuals are isolated from the cultures by transferring nylon mesh patches from the stock cultures and rinsing individuals off the patches with clean seawater. Gravid females are identified under a dissecting microscope.

E.3.6 Toxicity test procedure

A summary of the test protocol is shown in Table E.1, and schematically in Fig. E.3. Females are isolated from males and placed into culturing trays containing control sediment >7 days before the commencement of the test. This rest period increases the fecundity during the test and allows any existing embryos to develop and escape the marsupium. Males from the laboratory cultures are added to the culture of isolated females 1–2 days before the start of the test to mate with the isolated females. On the day before the test is started, each of the test sediment samples and the control sediment are homogenised within their storage containers. Pore water that has been separated from the samples during storage should be mixed back into the sediment using a non-toxic device, such as a stainless-steel spoon or Teflon® spatula. A 40 g aliquot of each of the sediments to be tested is added to four replicate 250 mL glass beakers for the reproduction test. The sediment is smoothed out and air pockets removed by gently tapping the beaker against the side of the hand to settle the contents. Overlying filtered seawater (30‰ or at the required salinity) is then added to each beaker to give a total volume of 220 mL. Filtered seawater is added by gentle and slow pouring down the side of the beaker to minimise mixing and re-suspension of the sediment. If monitoring of sediment chemistry is required, an additional beaker should be set up. Beakers are capped with acrylic covers and placed into a constant temperature cabinet or room at 21 ± 1°C overnight to equilibrate and to allow suspended particles to settle. The overlying water in each beaker is continuously gently aerated via glass pipette tips to maintain dissolved oxygen conditions.

On the following day (Day 0 of the test), overlying water from each of the test beakers is removed by gentle siphoning and is replaced by fresh filtered seawater at the required salinity. Following water renewals, the amphipods are sieved from their holding culture using a 600 µm mesh sieve and placed into a white plastic sorting tray (60 cm × 30 cm × 5 cm) in order to identify the sex of the amphipods. The test commences by adding 6 gravid females containing 1–2 day-old embryos (identified by microscopy) and 6 males to each beaker. Past tests have used 7 gravid females and 5 males (Simpson and Spadaro, 2011), but recently more robust results have been obtained using the same density of males and females. Amphipods are distributed using a wide-bore plastic pipette to plastic weighing

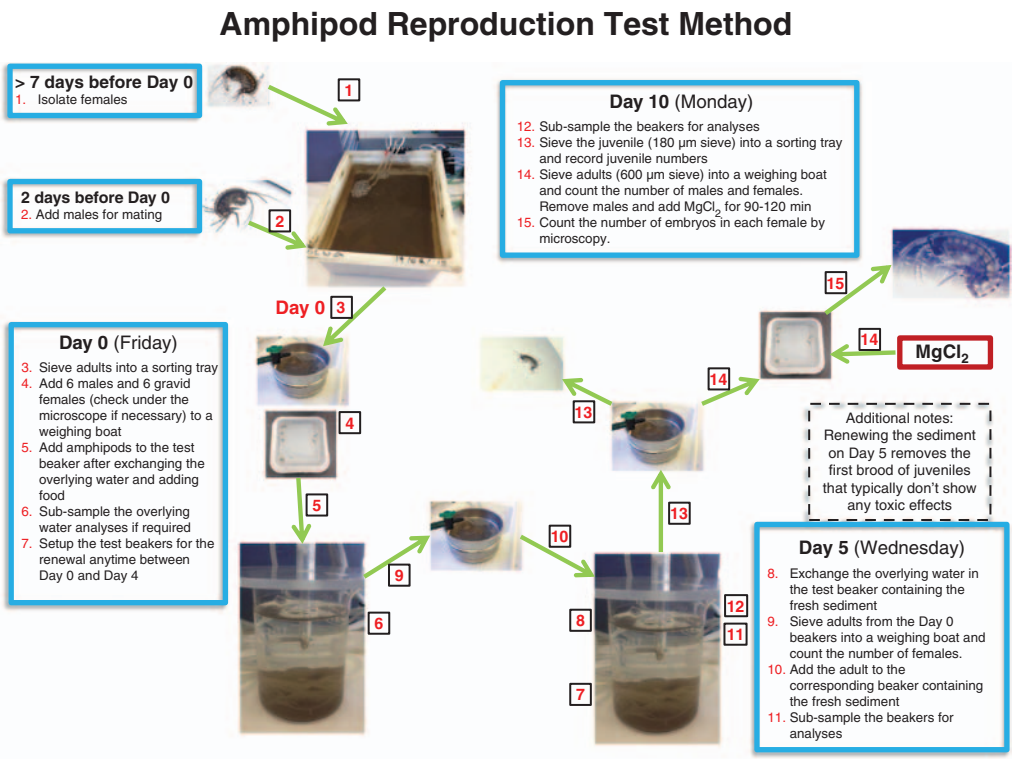

Figure E.3. Schematic diagram of the key steps in the 10-day whole-sediment sub-lethal (reproduction) test using the epibenthic amphipod *Melita plumulosa.*

boats before their addition to beakers to confirm the number and health (assessed by swimming behaviour) of all individuals added. Amphipods that are sluggish or appearing to behave atypically when sieved from the sediment are discarded. Once the amphipods have been added to the beakers, the test organisms are fed 0.5 mg of Sera Micron fish food per adult amphipod and the beakers are randomly positioned in the constant temperature cabinet or room at 21 ± 1°C. Beakers are capped with acrylic covers and the overlying water is continuously and gently aerated by slow bubbling for the test duration via glass pipette tips suspended 4 cm below the water surface to maintain ≥85% dissolved oxygen saturation without causing disturbance to the surface of the sediment. Lighting is on a 12-h light (3.5 µmol photons/s/m^2) : 12-h dark cycle throughout the test.

On each day throughout the test, beakers are checked to ensure that aeration is maintained and evaporation is minimal. On Days 3 and 7 the overlying water in the test beakers is exchanged with filtered seawater (this can be done more frequently if desired). Half (50%) of the overlying water is siphoned using tubing (8 mm in diameter or less) into a 180 µm sieve to ensure that no organisms are lost during the process, then the beakers are refilled with filtered seawater. After exchanging the overlying waters, the test organisms are fed 0.5 mg of Sera Micron fish food per adult amphipod. The test beakers are recapped and placed back into the cabinet.

On Day 4, the day before the sediment renewal, 40 g of test sediment is added to a clean unused 250 mL beaker, seawater is added and the beaker is placed in the cabinet to equilibrate.

On Day 5, the sediments are renewed in order to remove the first brood of juveniles which typically do not show any toxic effects because they were conceived in control sediment. On the day of renewal, the water in the renewal beakers is exchanged with filtered seawater. The test organisms in the test beakers are gently sieved (600 µm), placed in a plastic weighing boat and counted. This process is repeated until all test organisms have been found or all the test sediment has been examined. The recovered adult amphipods are

Table E.1. Summary of test conditions for the 10-day acute sediment toxicity test with the amphipod *Melita plumulosa*

Test type	Static test with sediment and renewals of overlying water
Test duration	10 days
Temperature	21 ± 1°C
Salinity	30 ± 1‰ (or as required)
pH	7.2 – 8.2
Ammonia	<1 mg/L
Light intensity	3.5 µmol photons/s/m^2
Photoperiod	12-h light : 12-h dark
Aeration	1 outlet with slow bubbling to maintain ≥85% dissolved oxygen saturation in overlying water throughout the test
Test chamber	250 mL glass beaker
Sediment weight	40 g
Overlying water volume	200 mL
Total test volume	250 mL
Age of test organisms	3–6 weeks old
No. of test organisms/test chamber	12 (6 males, 6 gravid females)
No. of replicate chambers/sample	4 (minimum) Additional replicate for chemical analysis sampling if required
Feeding	0.5 mg of Sera Micron per amphipod twice per week
Overlying water renewal	None if static test. Every 2 days for water renewal tests, but greater if desired
Control sediment	Uncontaminated sediment with similar physico-chemical parameters (grain size, pore-water salinity and pH) to the test sediment
Overlying water	Uncontaminated seawater collected from a clean site, filtered to 0.45 µm and diluted with deionised water to 30±1‰ or to the required salinity
Sediment sample weight required	1 kg (minimum 500 g)
Reference toxicant	Copper; Water-only exposure, duration = 96 h
Standard endpoint	Adult survival and reproduction
Test acceptability criteria	≥80% survival and 8–19 juveniles/gravid female in the control Physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) within acceptable limits throughout the test, and toxicant test results within specified limits

decanted from the weighing boat into the corresponding renewal beaker containing the same fresh sediment. Juveniles from the test beakers are discarded and not placed into the renewal beaker. The organisms are not fed on Day 5 in order to encourage them to forage in the sediments for food. The renewal beakers containing the adult amphipods are placed back into the cabinets.

On Day 7, as above, 50% of the overlying water is exchanged with filtered seawater and the organisms are fed.

After 10 days, the test is terminated. The contents of each beaker are wet-sieved through both 600 µm (adult) and 180 µm (juvenile) stainless-steel mesh screens, using filtered seawater to retain amphipods. Most of the overlying water is decanted through the sieve. The remaining water is swirled to suspend the top layer of sediment and this is decanted through the sieve. The majority of the amphipods should be in this top layer of the sediment. The remaining sediment is washed in stages through the sieve until all amphipods are accounted for or until all the sediment has been inspected. Material retained on both sieves is washed into separate sorting trays using control filtered seawater.

Juveniles from the 180 µm sieve and males from the 600 µm sieve are counted and the number recorded. All amphipods are collected using a wide-bore plastic pipette and placed in culture water for counting and to verify they are alive. The number of amphipods that are alive in each beaker is recorded, and the results expressed as percentage survival. Animals that are missing are presumed to have died and disintegrated and are counted as dead. Females and amplexed pairs (from the 600 µm sieve) are placed into a weighing boat containing 20 mL of 75 g/L $MgCl_2.9H_2O$ which acts as an anaesthetic. The anaesthetic requires 90–120 min to take effect. The embryos in each surviving female are counted under the microscope, and removed, if required using dissecting tools, in order to view all of the embryos.

The number of embryos (still present in the marsupium) and juveniles (escaped from the marsupium and found in the sediment) is divided by the original number of females and expressed as a percentage of the control.

E.3.7 Chemical and physico-chemical analyses

The monitoring of water quality and other desired parameters at the start, during and at the termination of tests should be undertaken in randomly selected replicates from each treatment (e.g. on Days 0, 2, 4, 5, 7, 9 and 10, depending on the project requirements and potential contaminants of concern) or, if disturbance is a concern, in an additional replicate test container set up per treatment. Physico-chemical parameters in the overlying water, including dissolved oxygen, pH, ammonia, salinity and temperature, are monitored to ensure that they remain within acceptable limits for quality control purposes. Sediment and pore-water (0.45 µm filtered, acid-washed filtered) samples may also be collected from the additional replicate test container at test termination for toxicant analysis. Overlying water and pore-water samples for metals analysis are typically acidified with concentrated high purity nitric acid (HNO_3) to a concentration of 0.2% and stored at 4°C until analysed. Samples for dissolved ammonia analyses may be preserved with sulfuric acid (below pH 2) and stored cold or frozen if analysis is not possible immediately, but analyses should ideally be undertaken as soon as possible (see Chapter 3 Sections 3.5.3, 3.7.1, and Chapter 4 Section 4.6.1).

E.3.8 Test acceptability and statistical analysis

Test conditions and physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) must remain within acceptable limits throughout the test.

Results of toxicity tests are reported in terms of the percentage reproduction in test sediments relative to reproduction in the control sediment. Toxicity is detected when the survival is <85% of the control (based on two standard deviations of control data, $n = 30$) and significantly less (t-test, $P < 0.05$) than the reproduction output observed in the control. Tests for significance between treatments and point estimate values (EC50, EC10) use ToxCalc for Microsoft Excel (Tidepool Software), or similar statistical software. A reference toxicant test should be undertaken periodically to monitor the sensitivity of the bioassay.

For quality assurance purposes, it is useful to chart total reproduction in all controls over time (Fig. E.2). If reference toxicants are tested, their results also should be within the expected range.

E.3.9 Reference toxicant tests

Because there are challenges with preparing reference sediments, water-only reference toxicant tests are recommended. The sensitivity of the amphipod to copper is well studied (Spadaro *et al*. 2008, Strom *et al*., 2011; Campana *et al*., 2012) and it is suggested that seawater be spiked with copper (as copper salt) for performing these tests. It is recommended that a 4-day juvenile survival test is conducted with a single copper exposure versus control, e.g. 100 µg Cu/L, as described by Spadaro *et al*. (2008). For more definitive tests, nominal total copper concentrations recommended are 0, 50, 100, 200, 400 µg/L. Dissolved copper concentrations should be measured at least three times throughout the tests to provide time-averaged concentrations for effects analysis.

All conditions for tests are the same as for the standard test described above, except that a minimum of three replicates are required for each test concentration. For definitive tests, concentrations tested must bracket the predicted LC50 from previous tests, and data obtained from reference toxicant tests are added to a cumulative chart in which the acceptable range for LC50 values is calculated (mean ± 2 s.d.).

Owing to ongoing changes that occur to contaminant-spiked sediments (Simpson *et al*., 2004), no standardised methods exist for preparing and testing contaminated sediments for test reference purposes. It is desirable that copper-spiked sediments are used, and methods for preparing this are described by Campana *et al*. (2012) and Simpson *et al*. (2004).

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Appendix F: Protocol for whole-sediment sub-lethal (reproduction) toxicity tests using the copepod *Nitocra spinipes* (harpacticoid)

David A. Spadaro and Stuart L. Simpson

F.1 Introduction

This appendix describes methods for testing the sub-lethal (chronic) adverse effects of potentially contaminated sediment on the reproduction of the epibenthic harpacticoid copepod species *Nitocra spinip*es (Ameiridae Boeck, 1865) (Fig. F.1). This species has a worldwide distribution and can adapt to a wide range of salinities and temperatures (Bengtsson, 1978).

Based on the method of Perez-Landa and Simpson (2011), this test measures the reproductive output of the copepod *N. spinipes* following exposure to undiluted test sediments over a 10-day period. The species is suitable for use in testing for the effects of contaminants in a full spectrum of sediment types (sand to silt, high to low organic carbon) (Ward *et al*., 2011). The sensitivity of reproduction of *N. spinipes* appears similar to that of the amphipod *Melita plumulosa* (see Appendix E), although the relative importance of the dissolved and particulate exposure pathways differs (Simpson and Spadaro, 2011).

Harpacticoid copepods (harpacticoids) are meiobenthic fauna (those typically 0.2–2.5 mm in size) that live within the sediment pore waters or adhering to the surface of individual sediment grains. Harpacticoids are the second most abundant metazoan invertebrates in sediments worldwide, making them an important component of the soft-bottom estuarine and marine benthic community. From an ecological perspective, harpacticoid copepods are important members of benthic communities because they feed on microbial and algal species and are a significant food source for other crustaceans and juvenile fish. As harpacticoids live closely within sediment, they may ingest fine sediment particles while feeding, potentially resulting in direct exposure to sediment-bound contaminants. These factors make harpacticoid copepods an appropriate species for sediment toxicity testing.

The harpacticoid copepod *Nitocra spinipes* was selected from a range of copepods that were cultured following isolation from sediments near Sydney, New South Wales (NSW), Australia. The species is relatively easily cultured in the laboratory, and reproduction

Figure F.1. Adult harpacticoid copepod *Nitocra spinipes* (non-gravid female, 0.5 mm length in photo, 0.2–0.8 mm range) (photograph: Daniel Ward).

occurs within acceptable timeframes for a wide range of sediment types (Perez-Landa and Simpson, 2011; Simpson and Spadaro, 2011; Ward *et al.*, 2011). Juvenile copepods hatch from the eggs as nauplius larvae that develop through multiple nauplius stages and then multiple sub-reproducing copepodite stages before taking the form of the mature adult copepod.

The development endpoints have been observed to be the most sensitive, with median effective concentration (EC50) values of 95 mg Cu/L for nauplii per gravid female and 101 mg Cu/L copepodites per gravid female, followed by the gravidity endpoint (144 mg Cu/L) and survival endpoint (347 mg Cu/L) (Perez-Landa and Simpson, 2011). Due to the similar sensitivity of the two development endpoints, total offspring is considered a suitable measure for use in routine toxicity testing. The test procedure described here is for a rapid test that provides an indication of the potential toxicity of estuarine and marine sediment samples to local biota.

F.2 Method basis and summary

Full life-cycle tests are usually defined as those that expose animals from one generation of eggs–nauplii (F0), through the various life stages to reproducing adults and to the next eggs–nauplii (F1). In some cases, the exposure of animals from hatching to reproductive maturity is also classified as a life-cycle test. Such life-cycle tests allow assessment of adverse effects of contaminant exposure on organism development and reproduction.

Challenges associated with conducting life-cycle tests often include the long life-cycles of many species, which may increase the variability arising from non-contaminant factors (for example, sediment properties), and the effects of confounding factors such as the source of food addition, and the cost of the tests compared with acute tests.

The full life-cycle of *N. spinipes* extends for 23–30 days (F0-nauplii to F1-nauplii) (Perez-Landa and Simpson, 2011; Ward *et al.*, 2011), which makes them highly suitable organisms for conducting short life-cycle tests. The females are iteroparous, producing several broods after only one mating encounter. For example, a single female may produce a brood of up to 30 nauplii every few days for several weeks after isolation from males. The development from nauplii to the intermediate stage copepodites usually takes 7–9 days, and a further 9–20 days are required for development into mature copepods, which can reach an average size of ~650 mm.

The 10-day copepod reproduction bioassay was adapted from the multistage approach described by Perez-Landa and Simpson (2011). By beginning the test with gravid females collected directly from the cultures (not pre-exposed to the test sediment), nauplii are released initially within 1–3 days, and then the iteroparous females become gravid again and drop further young during the test period. The total reproduction and survival of offspring – that is, total nauplii (first juvenile life-stage of the copepod) and copepodites (second juvenile life-stage) – is the endpoint after 10 days. The test provides similar sensitivity to multiple-generation test endpoints (development, gravidity; Perez-Landa and Simpson, 2011) and less variability in controls, making it more convenient for use in routine toxicity assessments.

The 10-day copepod reproduction bioassay is commenced with gravid females (Fig. F.2) isolated from laboratory cultures, and after 10 days the number of nauplii and copepodites are quantified.

The method is a static non-renewal bioassay. Sediments are homogenised, but not sieved, immediately before being added to test vials (0.5 g sediment per 1 cm diameter 10 mL vial, or 2 g of sediment per 50 mL centrifuge tube), with 5–6 replicates per sediment). Filtered seawater (pH 8.1, salinity 30‰) is added and each vial is incubated overnight to allow sediments to settle. On the following day, overlying water is replaced and 5 gravid females (3–5 weeks old) are randomly assigned to each vial. Treatments are fed twice a week during the test. After 10 days, the total offspring (number of nauplii and copepodites) in each vial is recorded by optical microscopy. For quality assurance purposes, a total reproductive output of 20–60 nauplii or copepodites per female is required in all controls for tests to be considered acceptable. The standard end point is the percentage reproduction of copepods exposed to the test sediment, relative to the controls.

The bioassays are undertaken at a temperature of 21 ± 1°C in an environmental chamber with a 12-h light : 12-h dark cycle (light intensity 3.5 µmol photons/s/m^2) for the test duration. For quality control purposes, physico-chemical parameters, including dissolved oxygen (>85% saturation is typically achieved by algal photosynthesis), pH (7.2–8.2), salinity (30 ± 2‰) and temperature (20–22°C) are monitored in the bulk seawater used at the beginning of the test period, and at test termination in a composite of the replicates

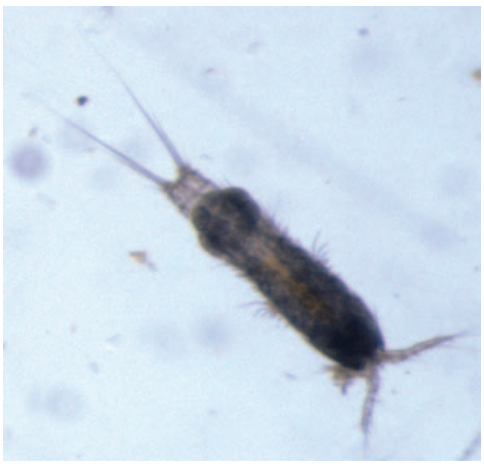

Figure F.2. Gravid female containing an egg sack at the posterior end of the copepod (0.5 mm length in photo, 0.2–0.8 mm range) (photograph: David Spadaro).

(because of the small volume of the test vessels) per sediment bioassay to ensure that they have remained within acceptable limits.

F.3 Method

F.3.1 Equipment

- Constant temperature chamber or temperature controlled room with 12-h light (3.5 µmol photons/s/m^2): 12-h dark cycle, maintained at 21 ± 1°C.
- Test chambers: Low volume method = 10 mL (1 cm diameter) plastic vial. High volume method = 50 mL centrifuge tube.
- Air supply and tubing (culturing only).
- Sterile filtered seawater (0.22 µm).
- Dissolved oxygen, pH and salinity/conductivity meters.
- Plastic and glass pipettes (wide bore).
- Stainless-steel spade or hand trowel to collect control sediment.
- Dissecting microscope.

F.3.2 Seawater

Seawater used for culturing, and as overlying water in toxicity tests is collected from an uncontaminated site and is filtered to 0.45 µm. In addition to filtering, seawater used in the culturing of the copepods is sterilised by autoclaving (but in some cases the removal of egg and juvenile organisms by further filtering to 0.22 µm may be adequate). The salinity of filtered seawater is typically 33–35‰ and is adjusted to a lower salinity (as required) by the addition of deionised water (e.g. Milli-Q® water).

F.3.3 Control sediment

Sediments used for culturing the copepod *N. spinipes* and as the control sediment in toxicity tests may be collected from uncontaminated estuarine intertidal areas. Suitable locations should have low concentrations of metal and organic contaminants. Control sediments can be collected using a stainless-steel spade or hand trowel, and press sieved (wearing gloves) through a 2 mm plastic mesh sieve on site to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in cooler boxes with ice or cooler blocks and returned to the laboratory and stored at 4°C for a maximum of 2 months. Sediment is placed at room temperature (21 ± 2°C) for a minimum of 24 h before being used, to allow it to equilibrate to the test and culturing temperature. As reproduction in test sediments is compared to reproduction in control sediments in the toxicity test, it is crucial that the control sediment has not only low contaminant levels but also similar physico-chemical parameters to the test sediment, including grain size and pore-water salinity. A range of particle sizes for the control sediments can be achieved by adding clean sand.

F.3.4 Test sediment

Test sediment should be collected and stored in containers made of inert materials to prevent contamination. Sediment should be chilled when collected, shipped on ice and stored in the dark in a refrigerator at 4°C. It should not be frozen nor allowed to dry out and should be used in tests as soon as possible and within 8 weeks of receipt. Before use, test sediment is held at room temperature (21 ± 2°C) for a minimum of 24 h to allow it to equilibrate to the test temperature.

F.3.5 Test organisms

Collection, handling and culturing of copepods

Copepods used in tests are obtained from laboratory-maintained cultures. Stock cultures were originally established from *N. spinipes* isolated from an estuarine environment on the upper Port Hacking River (NSW, Australia). The species is widely distributed internationally, and other sites should readily be found elsewhere in Australia or worldwide (Bengtsson, 1978). To isolate copepods, sediments are sieved using a 250 μm mesh to remove large particles and debris and then adult copepods are isolated from the <250 μm sediment under a light microscope using a glass pipette. Initial cultures are started using 100–200 individuals. Copepods whose morphology cannot be matched to *N. spinipes* are removed and discarded.

Copepod cultures are established in clean plastic containers (20 cm × 14 cm × 10 cm) with a 0.5–1 cm layer of a mixture of sterilised silty sediment and clean sand (~50% particle size <63 μm) with bi-weekly water changes (0.45 μm filtered seawater). To expand cultures, the adult copepods are sieved (50 μm nylon sieve) and used to commence new cultures, and any copepods that do not match the morphology of the selected species (including nauplii and copepodites) are again discarded during this process to purify the cultures.

The sediment used for cultures is sterilised (e.g. by autoclaving for 30 min at 120°C) to eliminate pre-existing meiofauna, particularly nematodes that may otherwise multiply to large numbers and compete with copepods for food resources.

During culturing, copepod cultures are fed 10 mL of a 1:1 mix of the algae *Tetraselmis* sp. and *Cryptomonas* sp. (total concentration 1×10^7 cells/mL) twice weekly in established cultures (>3 weeks old) or 5 mL in developing cultures (<3 weeks old). (Note: other food sources, such as sieved Sera Micron fish food and a brown diatom, are often also suitable and may be trialled.) New copepod cultures are initiated every 2–3 weeks by transferring 100 gravid female copepods from the existing cultures to new culture containers.

Adult copepods are removed from cultures using a plastic Pasteur pipette to gently suck up aliquots from the surface sediment. Each aliquot is passed through a 180 μm sieve, retaining adults while letting most of the sediment, debris, and juveniles pass through the mesh back into the culture container. Adults are transferred from the sieve (using clean seawater) into a Petri dish and placed under a light microscope (maximum magnification 369×), and individual copepods are sorted, based on maturity, sex, and presence of egg sacs, using a glass Pasteur pipette.

F.3.6 Toxicity test procedure

A summary of the test protocol is shown in Table F.1, and schematically in Fig. F.3. On the day before the test is started, each test sediment sample and the control sediment are homogenised within their storage containers. Pore water that has separated from the samples during storage should be mixed back into the sediment using a nontoxic device, such as a stainless-steel spoon or Teflon® spatula. A 0.5 g aliquot of each of the sediments to be tested is added to 5–6 replicate 10 mL plastic vials. The sediment is smoothed out and air pockets removed by gently tapping the vial against the side of the hand to settle the contents. Overlying filtered seawater (30‰ or at the required salinity) is then added to each vial to give a total volume of 10 mL. Filtered seawater is added by gentle and slow pouring down the side of the vial to minimise mixing and resuspension of the sediment. Vials are capped and placed into a constant temperature chamber or room at 21 ± 1°C overnight to equilibrate and to allow suspended particles to settle. The test vials are not aerated because dissolved oxygen is adequately maintained by the addition of algae also used as a food source.

The day before the addition of the test organisms, the cultures are fed, which promotes gravidity in the females. On the following day (Day 0), the water overlying the sediment in each of the test vials is removed by gentle syringing and is replaced by fresh filtered seawater at the required salinity. Following these water renewals, the test commences when the gravid females are identified and randomly assigned to each replicate vial. Several steps are required to achieve this. Firstly, a plastic pipette is used to draw up the sediment–water interface (top few mm) in the culture and dispense this onto a Petri dish for examination under the microscope. Secondly, gravid females with large oval egg sacs are selected and are moved into a droplet of clean seawater on a separate Petri dish to wash away any culturing sediment. Finally, from this Petri dish, 4 or 5 females (same number for all replicates) are pipetted into each test vial and the pipette is rinsed with seawater to ensure all the females have been removed from the pipette into the test vial. Ideally the test uses 5 gravid females per replicate. Four in each replicate can be used if necessary, but the 'test acceptability criteria' for reproduction output in controls (see Section F.3.8) is more frequently achieved using 5.

Once the copepods have been added and fed, the vials are randomly positioned on a vial rack and placed in the constant temperature chamber or room at 21 ± 1°C. Lighting is on a 12-h light (3.5 µmol photons/s/m^2) : 12-h dark cycle throughout the test.

The copepods are fed by adding 150 µL of feeding stock (0.15 mg <63 µm Sera Micron, 10 mL (1 × 10^5 cells per mL) of *Tetraselmis* sp. and 10 mL (1 × 10^5 cells per mL) of *Cryptomonas* sp.) twice a week during the test (i.e. Days 0, 3 and 7).

On Day 10 the test is terminated. Overlying water (5 mL) from each replicate is checked under the microscope for copepodites and retained for metal analysis and measures of

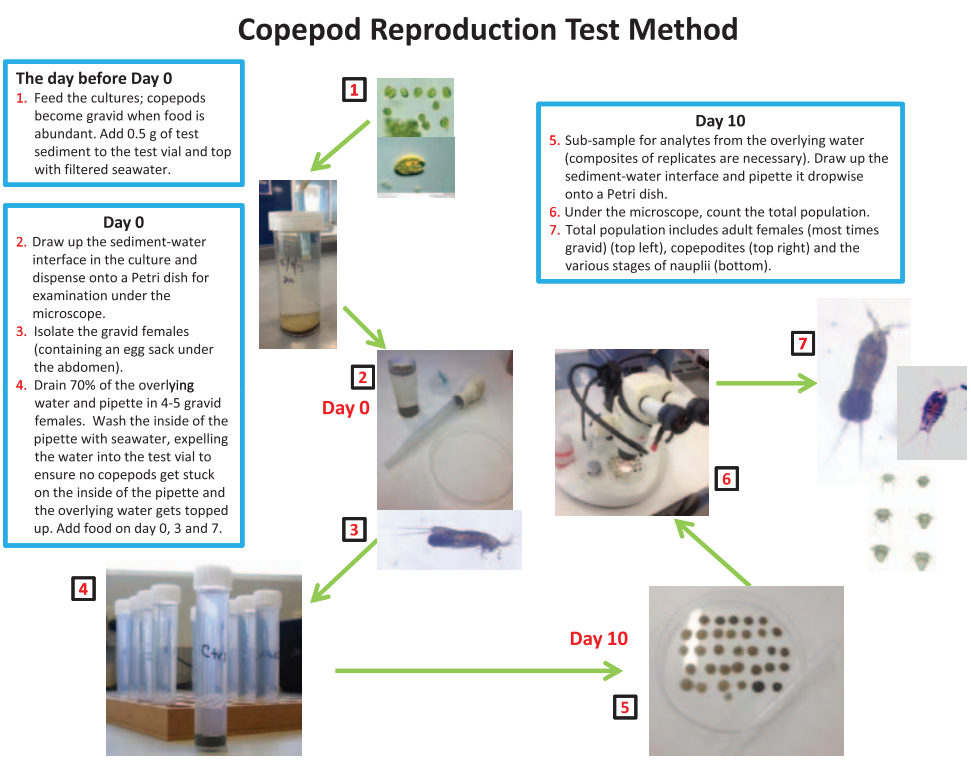

Figure F.3. Schematic diagram of the 10-day whole-sediment sub-lethal (reproduction) test using the copepod *Nitocra spinipes*.

Table F.1. Summary of test conditions for the 10-day sub-lethal (reproduction) toxicity tests using the copepod *Nitocra spinipes* in sediments

Test type	Static non-renewal	
Test duration	10 days	
Temperature	21 ± 1°C	
Salinity	30 ± 2‰ (or as required)	
pH	7.2 – 8.2	
Ammonia	<1 mg/L	
Light intensity	3.5 µmol photons/s/m^2	
Photoperiod	12-h light : 12-h dark	
Aeration	No bubbling required due to the small amount of sediments and small organisms. Desirable to maintain ≥85% dissolved oxygen saturation in overlying water throughout the test	
	Low volume method	**High volume method**
Test chamber	10 mL polycarbonate vial (1 cm diameter)	50 mL centrifuge tube
Sediment weight	0.5 g	2 g
Overlying water volume	~9 mL	45 mL
Total test volume	10 mL	50 mL
Age/size of test organisms	3–5 week gravid females	
No. of test organisms/ test chamber	5	
No. of replicate chambers/sample	5–6	
	Further replicates may be added for additional chemical analysis sampling if required	
Feeding	150 µL of feeding stock (0.15 mg <63 µm Sera Micron, 10 mL of 1×10^5 cells per mL of *Tetraselmis* sp. (strain CS-87) and 10 mL of 1×10^5 cells per mL of *Cryptomonas* sp.) twice a week during the test	
Overlying water renewal	None during test (static)	
Control sediment	Uncontaminated sediment with similar physico-chemical parameters (grain size, pore-water salinity and pH) to the test sediment	
Overlying water	Uncontaminated seawater collected from a clean site, filtered to 0.45 µm and diluted with deionised water to 30 ± 1‰ or to the required salinity	
Sediment sample weight required	50 g	
Reference toxicant	none	
Standard endpoint	Reproductive output (nauplii and copepodites produced during the test)	
Test acceptability criteria	>20 juveniles per female in controls	
	Physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) within acceptable limits throughout the test; reference toxicant test results within specified limits	

physico-chemical parameters. The test vial is gently agitated to resuspend the upper layers of the sediment, creating a slurry. This slurry is pipetted dropwise onto a large Petri dish. This allows efficient and thorough counting of the replicate population. The number of juvenile copepods in that vial is recorded, and the results are expressed as the number of juveniles per female.

F.3.7 Chemical and physico-chemical analyses

Physico-chemical parameters in the overlying water, including dissolved oxygen, pH, ammonia, salinity and temperature, are monitored to ensure that they remain within acceptable limits for quality control purposes. The monitoring of water quality and other parameters of interest at the start, during and at the termination of tests should be undertaken in randomly selected replicates from each treatment for the high volume method, or in an additional replicate test container set-up per treatment for the low volume method (this container may be considerably larger but ideally proportional to the small copepod test vessel). Sediment and pore-water (0.45 μm filtered, acid-washed filtered) samples may also be taken from the additional replicate test container at test termination for toxicant analysis.

Overlying water and pore-water samples for metals analysis are typically acidified with concentrated high purity nitric acid (HNO_3) to a concentration of 0.2% and stored at 4°C until analysed. Samples for dissolved ammonia analyses may be preserved with sulfuric acid (below pH 2) and stored cold or frozen if analysis is not possible immediately, but analyses should ideally be undertaken as soon as possible (see Chapter 3 Sections 3.5.3, 3.7.1 and Chapter 4 Section 4.6.1).

F.3.8 Test acceptability and statistical analysis

Test conditions and physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) must remain within acceptable limits throughout the test.

Results of toxicity tests are reported in terms of the percentage reproduction in test sediments relative to reproductive output in the control sediment. Reproductive output of the copepods is expressed as the number of juveniles per surviving female. Toxicity is detected when the reproductive output is <75% of the control (based on two standard deviations of control data, $n = 30$) and significantly less (t-test, $P < 0.05$) than the reproductive output observed in the control. Tests for significance between treatments and point

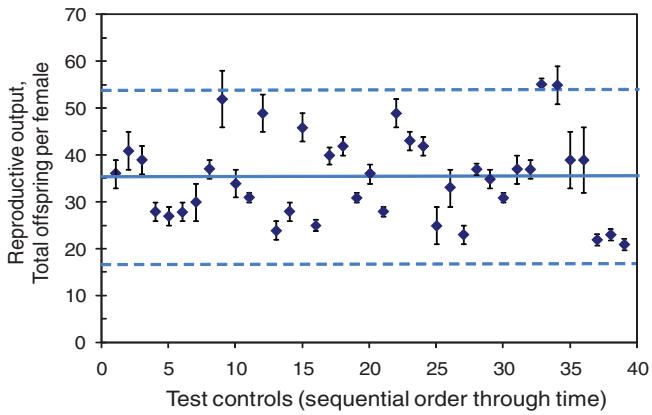

Figure F.4. Example of control chart for total reproductive output for quality assurance purposes, showing the mean (solid line) and standard deviations of the mean (dashed lines).

estimate values (EC50, EC10) use ToxCalc Version 5.0.23 (Tidepool Software) or similar statistical software. A reference toxicant test should be undertaken periodically to monitor the sensitivity of the bioassay.

For quality assurance purposes, it is useful to chart total reproductive output in all controls over time (e.g. Fig. F.4).

Similarly, it is useful to monitor the sensitivity to contaminants, which can be done using reference toxicant tests or through comparison with other sub-lethal tests and sediment contaminant gradients (Simpson and Spadaro, 2011).

F.3.9 Reference toxicant test

A water-only reference toxicant test is recommended over a whole-sediment test because of its greater ease and the reduced time spent in preparing and conducting the test, and because of the greater consistency in the response of copepods observed in replicate tests. For water-only tests, it is recommended that a 7-day or 10-day development test is conducted (Perez-Landa and Simpson, 2011). The sensitivity of the copepod to dissolved copper is well studied (Perez-Landa and Simpson, 2011) and it is suggested that seawater be spiked with copper (as copper salt) for performing these tests. Nominal total copper concentrations recommended are 0, 50, 100, 200, 300, 500 μg/L.

All conditions for reference toxicant tests are the same as for the standard test described above, except a minimum of 3 replicates is required for each test concentration. Concentrations tested must bracket the predicted LC50 value from previous tests, and data obtained from reference toxicant tests are added to a cumulative chart in which the acceptable range for LC50 values is calculated (mean ± 2 s.d.).

Owing to ongoing changes that occur in contaminant-spiked sediments (Simpson *et al*., 2004), no standardised methods exist for preparing and testing contaminated sediments for test reference purposes. If this testing is desirable, it is suggested that copper-spiked sediments be used, as described in Campana *et al*. (2012) and Simpson *et al*. (2004).

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Appendix G: Protocols for 10-day whole-sediment lethality toxicity tests and 30-day bioaccumulation tests using the deposit-feeding benthic bivalve *Tellina deltoidalis*

David A. Spadaro and Stuart L. Simpson

G.1 Introduction

This appendix describes methods for testing the short-term (acute) adverse effects of potentially contaminated sediment on the survival of, and longer-term bioaccumulation by, the estuarine bivalve *Tellina deltoidalis* (Fig. G.1). This species occurs in estuaries and coastal lagoons from southern Queensland to Tasmania and southern Western Australia. The methods represent robust approaches for assessing acute lethality and bioaccumulation endpoints for whole sediments.

Tellina deltoidalis lives buried in the top 2–20 cm of silty or sandy sediments and, like other tellinids, is a deposit feeder, stirring the sediment surface with its siphon while feeding (Willan, 1998; Ponder *et al*., 2000; Campana *et al*., 2013). Metal exposure studies indicate that both aqueous and sediment-bound contaminants contribute to accumulation and toxicity (King *et al*., 2005; Simpson and King, 2005; Strom *et al*., 2011; Campana *et al*., 2013).

Based on the method of King *et al*. (2010), this protocol describes methods to assess 10-day (acute) lethality, and 30-day bioaccumulation, during exposure of *T. deltoidalis* to undiluted test sediments. The species is suitable for testing for the effects of contaminants in a full spectrum of sediment types (0–100% <63 µm particle size) and a broad salinity range (10–34‰) (King *et al*., 2004). It can also be used in bioaccumulation studies (Simpson *et al*., 2006, 2012; Atkinson *et al*., 2007; Campana *et al*., 2015; Taylor and Maher, 2013, 2014a,b), sub-lethal growth tests (Campana *et al*., 2013), and in *in situ* tests on sediments in the field (Belzunce *et al*., 2015).

Bivalves are an abundant and important component of the soft-bottom estuarine and marine benthic community. Many infaunal species, including *Tellina deltoidalis*, feed on suspended particulate matter in the water column (filter-feeding) as well as directly ingesting sediment particles (deposit-feeding). Their food includes organic materials such as bacteria, algae and other plant detritus, but feeding behaviours do not exclude inorganic sediment materials. They are therefore potentially exposed to both aqueous and sediment-bound

Figure G.1. Adult bivalve *Tellina deltoidalis* (photograph: David Spadaro).

contaminants. Bivalves are a principal prey item of many birds, fish and larger invertebrates and many species accumulate high levels of contaminants in their tissues. They have the potential for trophic transfer of contaminants from sediments and waters to higher trophic levels. The ecological importance of bivalves, their wide distribution and high abundance, their ease of handling and their relative sensitivity to contaminated sediments, make them appropriate species for sediment toxicity testing.

Tellina deltoidalis was selected as a preferred tests species over other benthic bivalves (*Mysella anomala, Soletellina alba, Spicula trigonella*) after consideration of both the ease of collection of this species and its sensitivity to common contaminants (King *et al*., 2004; Batley and Simpson, 2009; Strom *et al*., 2011).

The procedure described here is for a relatively rapid standard toxicity test that provides an indication of the potential acute toxicity and bioaccumulation of contaminants in estuarine and marine sediment samples for local biota. The acute lethality test is not intended to exactly simulate the exposure of benthic bivalves under 'natural' conditions but is conducted to obtain information on the immediate effects of a short-term exposure to test sediment under specific environmental conditions. The bioaccumulation of contaminants in the body tissues of exposed bivalves is useful for determining the biological availability of contaminants and the likely routes of exposure and uptake of contaminants by the bivalve. Tests can also be developed with growth rate endpoints; for example, 30–40 day growth rate tests (Campana *et al*., 2013).

G.2 Method summaries

The 10-day survival test uses control sediment and one or more test sediments. For each sediment it is usual to have a minimum of 4 replicates, with each replicate containing 10–15 bivalves (young adults, 5–10 mm in length). In the test, 200 g of sediment with overlying water to 900 mL is added to a 1 L beaker. The response of bivalves during 10 days in the test sediment is compared with their response in a control (reference) sediment. The 30-day bioaccumulation test protocol follows that of the 10-day toxicity test but is extended to 30 days, and surviving organisms are retained for analysis of tissues (King *et al*., 2010). The concentration of contaminants in the soft body tissues of bivalves is determined in surviving bivalves at the termination of tests.

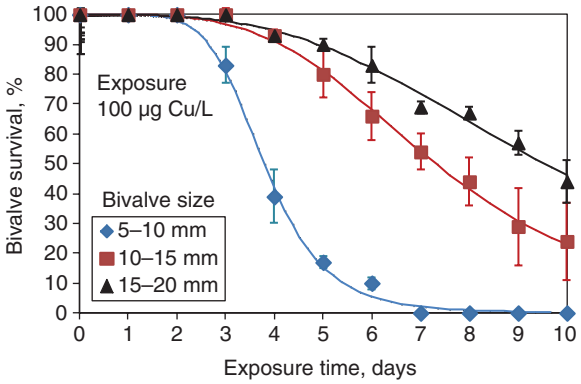

Figure G.2. Survival of varying size classes of adult *Tellina deltoidalis* during 10-day exposures to dissolved copper (Strom *et al.*, pers. comm.).

The standard endpoint is percentage survival in the test sediment relative to the controls. Additional endpoints that may be used include post-exposure recovery, post-exposure reburial, and accumulation of contaminants in the bivalve's soft body tissues and/or shell. The size of the bivalves reflects their age: large adult organisms are more tolerant to toxicants than young adults (Fig. G.2).

G.3 Method

G.3.1 Equipment

- Constant temperature chamber or temperature controlled room with 12 h light (3.5 µmol photons/s/m^2): 12 h dark cycle, maintained at the desired temperature (e.g. 21 ± 1°C).
- Test chambers: 1 L glass beakers.
- Diluent/control filtered seawater (0.45 µm).
- Dissolved oxygen, pH and salinity/conductivity meters.
- Plastic or stainless-steel sieve with 2 mm mesh.
- Plastic containers (16 cm × 12 cm × 4 cm) for field collections.
- Plastic holding trays (40 cm × 30 cm × 10 cm) for laboratory holding.
- Small plastic shovel, spoon, spade or hand trowel to collect control sediment.
- Large shovel to collect sediment large quantities of sediments to be sieved for bivalves.
- Cooler box with ice or cooler blocks.

G.3.2 Seawater

Seawater used for acclimatising bivalves and as overlying water in toxicity tests is collected from an uncontaminated site and filtered to 0.2–1 µm. The salinity of filtered seawater is typically 33–35‰ and is adjusted to a lower salinity (as required) by the addition of deionised water (for example, Milli-Q® water).

G.3.3 Control sediment

Control sediment used in toxicity tests is collected from the site of bivalve collection or from an uncontaminated area with sediment that has properties within the geochemical requirements

of the species. Control sediment is collected using a plastic spoon and is press-sieved (wearing gloves) through a 2 mm plastic mesh sieve on-site to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in a cooler box with ice or cooler blocks and returned to the laboratory and stored at 4°C for a maximum of 2 months. Sediment is held at room temperature (e.g. 21 ± 2°C) for a minimum of 24 h before being used, to allow it to equilibrate to the test and culturing temperature. As survival in test sediments is compared to survival in control sediments in the toxicity test, it is important that the control sediment has not only low contaminant concentrations but also similar physico-chemical parameters to the test sediment, including grain size and pore-water salinity.

G.3.4 Test sediment

Test sediments should be collected and stored in containers made of inert materials to prevent contamination. Sediment should be chilled when collected, shipped on ice and stored in the dark in a refrigerator at 4°C. It should not be frozen nor allowed to dry out and tests should commence as soon as possible and ideally within 8 weeks of receipt. Prior to its use, test sediment is held at room temperature (e.g. 21 ± 2°C) for a minimum of 24 h to allow it to equilibrate to the test temperature.

G.3.5 Test organisms

Collection and handling of bivalves

Bivalves used in tests are obtained from field populations collected from estuarine sand and mud flats. Bivalves are collected at low tide, using a large shovel to transfer 5–10 kg of surface sediment (top 10–15 cm) to a 2 mm or 4 mm mesh plastic sieve, and then using shallow water to assist the sieving away of the sediment to expose organisms. Only young adults of a uniform size and age (5–10 mm in length) and having a shell without a crack or hole are collected. They are placed into clean plastic containers (16 cm × 12 cm × 4 cm) with seawater and a 3 cm thick layer of sediment, collected *in situ*. Containers with bivalves are transported back to the laboratory in a portable icebox, maintained at ambient temperature or lower. Bivalves are kept in a temperature-controlled laboratory (e.g. 21 ± 2°C) in clean plastic holding trays (40 cm × 30 cm × 10 cm) containing 1.1 mm-sieved sediment to a depth of 3 cm and ~10 cm of overlying water (filtered seawater) at a salinity of 30‰. A maximum of 150 bivalves may be kept per tray without overcrowding. Overlying water in trays is continuously gently aerated and trays are covered with foil to minimise light disturbances to the bivalves. The overlying water in the holding trays is exchanged every 2 days to avoid the build-up of ammonia.

Bivalves are held in the laboratory for a minimum of 2 days and a maximum of 2 weeks before being used in tests. If required (i.e. test salinity is different from 30‰), bivalves are slowly acclimatised to the test salinity over this period, by increasing or decreasing the salinity of the overlying water in aquaria by 1–2‰ per day. At least 75% of the water is removed each day and replaced with filtered seawater at the appropriate salinity (prepared by adding deionised water to natural filtered seawater (35‰) each day). Animals are isolated from the holding sediment by gentle sieving through 2 mm plastic sieves immediately before test commencement.

G.3.6 Toxicity test protocol

A summary of the test protocol is shown in Table G.1. On the day before the test is started, each test sediment sample is homogenised within its storage container. A 200 g aliquot of

each of the sediments to be tested is added to each of 4–5 replicate 1 L glass beakers. The sediment is smoothed and air pockets removed by gently tapping the beaker against the side of the hand to settle the contents. Overlying filtered seawater (30‰ or at the required salinity) is then added to each beaker to give a total volume of 900 mL. Filtered seawater is added by pouring it gently and slowly down the side of the beaker to minimise mixing and re-suspension of the sediment. Beakers are capped with Perspex® covers and placed into a constant temperature chamber or room (e.g. 21 ± 1°C) overnight to allow suspended particles to settle and equilibrium to be established between the sediment and overlying water. The overlying water in each beaker is continuously gently aerated to maintain dissolved oxygen conditions. If monitoring of sediment chemistry is required, additional beakers with sediment and bivalves should be set up.

On the following day, Day 0, overlying water from each of the test beakers is removed by gentle siphoning and replaced by fresh filtered seawater at the required salinity. Following water renewals, the test commences when a total of 10–15 bivalves are randomly assigned and added to each beaker. Bivalves are sieved from holding trays and checked for cracks or holes in their shells immediately before being added to test beakers at the start of the test. Once the bivalves have been added, the beakers are again covered and randomly positioned in the constant temperature chamber or room (e.g. 21 ± 1°C) with constant gentle aeration to the overlying water.

On each day throughout the test, the beakers are checked to ensure that there is adequate aeration, where possible any dead animals and shells are removed from the beakers and counted (as part of survival data). Observations to assess bivalve behaviour and location are also made daily if required. Greater than 90% of the overlying water is exchanged every second day, and dissolved metals (if required), ammonia, temperature, pH, salinity and dissolved oxygen are measured before water renewal throughout the 10-day test. When refilling beakers with filtered seawater, care is needed not to resuspend the sediment.

In the 10-day survival test, the test is terminated by sieving the contents of each beaker through a 2 mm mesh plastic sieve to retain bivalves at the end of day 10. All bivalves are collected using Teflon® tweezers and placed in filtered seawater in small plastic trays (16 cm × 12 cm × 2 cm) for counting and to verify they are alive. The number of surviving bivalves in each beaker is recorded, and the results expressed as percentage survival. The standard endpoint is percentage survival in the test sediment relative to the control (reference) sediment after 10 days.

The 30-day bioaccumulation test protocol follows that of the 10-day toxicity test, but is extended to 30 days, and surviving organisms are retained for analysis of tissues. Surviving bivalves are left for 24 h in beakers containing seawater to allow clearance of their gut passages and the removal of sediment particles trapped within the organisms' shells. This is to ensure that the body tissue concentrations represent only the contaminants that have been assimilated (King *et al*., 2005). After the depuration period, bivalves are dissected with Teflon®-coated blades to separate the shell from the soft body tissue, and the soft tissues from each individual bivalve are placed in pre-weighed 70 mL acid-washed polycarbonate vials and frozen for tissue analyses.

G.3.7 Chemical and physico-chemical analyses

Water quality and other desired parameters should be monitored at the start of, during, and at the termination of tests, in randomly selected replicates from each treatment, or, if disturbance is a concern, in an additional replicate test-container set up per treatment. Physico-chemical parameters in the overlying water, including dissolved oxygen, pH,

ammonia, salinity and temperature, are monitored to ensure that they remain within acceptable limits for quality control purposes. Sediment and pore-water (0.45 µm filtered, acid-washed filtered) samples may also be collected from the additional replicate test container at test termination for toxicant analysis. Overlying water and pore-water samples for metals analysis are typically acidified with concentrated high purity nitric acid (HNO_3) to a concentration of 0.2% and stored at 4°C until analysed. Samples for dissolved ammonia analyses should, ideally, be analysed as soon as possible, but may be preserved with sulfuric acid (below pH 2) and stored cold or frozen if analysis is not possible immediately (see Chapter 3 Sections 3.5.3, 3.7.1, and Chapter 4 Section 4.6.1).

G.3.8 Test acceptability and statistical analysis

Test conditions and physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) must remain within acceptable limits throughout the test.

Results of toxicity tests are reported in terms of the percentage survival in test sediments relative to survival in the control sediment. Toxicity is detected when the survival

Table G.1. Summary of test conditions for the 10-day acute sediment toxicity test with the bivalve *Tellina deltoidalis*

Test type	Static non-renewal
Test duration	10 days
Temperature	21 ± 1°C
Salinity	30 ± 1‰ (or as required)
pH	7.8–8.2
Light intensity	3.5 µmol photons/s/m^2
Photoperiod	12-h light : 12-h dark
Aeration	1 outlet with slow bubbling to maintain ≥85% dissolved oxygen saturation in overlying water throughout the test
Test chamber	1 L glass beaker
Sediment weight	200 g
Overlying water volume	700 mL
Total test volume	900 mL
Size of test organisms	5–10 mm length
No. of test organisms/ test chamber	10–15
No. of replicate chambers/ sample	4 (minimum)
Feeding regime	None during the test
Overlying water renewal	None during the test (static)
Control sediment	Uncontaminated sediment with similar physico-chemical parameters (grain size, pore-water salinity and pH) to the test sediment
Overlying water	Uncontaminated seawater collected from a clean site, filtered to 0.45 µm and diluted with deionised water to 30 ± 1‰ or to the required salinity
Sediment sample weight required	1.5–3 kg

Table G.1. (Continued)

Reference toxicant	Cu; Water-only exposure, duration = 96 h time to death; or 3-, 5-, and 7-day LC50 values
Standard endpoint	Survival
Alternative endpoints	Post-exposure recovery in filtered seawater Post-exposure reburial in control sediment Contaminant tissue concentrations
Test acceptability criteria	≥80% survival in controls, physico-chemical parameters (dissolved oxygen, pH, salinity and temperature) within acceptable limits throughout the test

is <80% of the control and significantly less (*t*-tests $P < 0.05$) than the survival observed in the control. Tests for significance between treatments and point estimate values (EC50, EC10) use ToxCalc for Microsoft Excel (Tidepool Software), or similar statistical software. A reference toxicant test should be undertaken periodically to monitor the sensitivity of the bioassay.

For quality assurance purposes, it is useful to chart or keep a record of total survival in all controls over time (survival in controls is always ≥90%). If a reference toxicant test is run, the results should be within the expected range.

G.3.9 Reference toxicant test

A water-only reference toxicant test is recommended rather than a whole-sediment test because of the greater ease and reduced time spent in preparing and conducting the test, and because of the greater consistency in the response of bivalves observed in replicate tests. The sensitivity of the bivalve to copper is well studied (Strom *et al*., 2011; Campana *et al*., 2013) and it is suggested that seawater be spiked with copper (as copper salt) for performing these tests. For the reference toxicant test it is recommended that a 7-day time-to-death survival test is conducted and the time (duration) until 50% lethality occurs (LT50) is calculated and charted (as in Fig. G.2). For water-only exposures to dissolved copper, Strom *et al*. (2011) determined 3-, 5-, and 7-day LC50 values for *T. deltoidalis* as, respectively, 208, 102, and 9.9 μg Cu/L. While these results confirm the bivalves' sensitivity to dissolved copper, additional stress caused by the absence of a sediment substrate will also have contributed to this sensitivity.

For definitive tests, nominal total copper concentrations recommended are 0, 50, 100, 200, 400 μg/L (Strom *et al*., 2011). Dissolved copper concentrations should be measured at least three times throughout the tests to provide time-averaged concentrations for effects analysis.

All conditions for the reference toxicant test are the same as for the standard test described above, except that a minimum of 3 replicates is required for each test concentration. For definitive tests, concentrations tested must bracket the predicted LC50 value from previous tests. Data obtained from reference toxicant tests are added to a cumulative chart in which the acceptable range for LC50 values are calculated (mean ± 2 s.d.).

Owing to ongoing changes that occur in contaminant-spiked sediments (Simpson *et al*., 2004), no standardised methods exist for preparing and testing contaminated sediments for test reference purposes. If this testing is desirable, it is suggested that copper-spiked sediments be used, as described in Simpson *et al.* (2004) and Campana *et al*. (2013).

G.3.10 Accumulation of contaminants in the body tissues (30-day test only)

Accumulation in the soft tissue component is usually analysed, but the shell may also be analysed. Methods for metals and organic contaminant analyses in tissues will differ and, in the case of organics, may need to be undertaken on wet or freeze-dried tissues rather than oven-dried tissues. Soft tissues from single bivalves or composites may be rinsed with deionised water to avoid salt interferences with analytical equipment. The tissues (with excess water removed by shaking) are transferred to a pre-weighed acid-washed polycarbonate vial. The wet weight of each soft tissue is determined using an analytical balance accurate to 0.05 mg. However, as results can be reported on a dry or wet weight basis, a representative tissue sample must be used for moisture analysis. In the case of metals analyses, tissues may either be dried in an oven at 60°C for a minimum of 24 h, or freeze-dried. After cooling at room temperature in a desiccator, each vial (and dried tissue) is reweighed using an analytical balance accurate to 0.05 mg and the dry weight of the bivalve soft tissue is calculated.

Tissues are analysed using standard methods. For quality control purposes, blank, replicate and certified reference materials must always be included.

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Appendix H: Protocol for 10-day whole-sediment sub-lethal and acute toxicity tests using the freshwater chironomid *Chironomus tepperi*

Anu Kumar and Stuart L. Simpson

H.1 Introduction

This appendix describes methods for testing the lethal and sub-lethal (chronic) adverse effects of potentially contaminated sediment on the survival, growth and adult emergence of the chironomid species *Chironomus tepperi* (Arthropoda, Dipteran, Chironomidae) (Fig. H.1). This is a red bloodworm/non-biting midge species that is native to south-eastern Australia. The method described represents a sensitive and robust approach for undertaking whole-sediment toxicity tests on freshwater sediments with acute and sub-lethal, chronic test endpoints.

Chironomids are widely distributed in many ecosystems, and have high abundances in many freshwater environments. They inhabit lakes, ponds and streams and typically live in muddy-bottom littoral habitats. *C. tepperi* Skuse is an endemic Australian chironomid that rapidly colonises newly inundated environments (Martin and Porter, 1978; Stevens, 1993).

Chironomus tepperi is suitable for use in testing for the effects of contaminants in a full spectrum of sediment types (sand to silt, high to low organic carbon), and over a wide range of temperatures. The multiple lethal and sub-lethal endpoints are sensitive and robust for a range of contaminants. This toxicity test is based on protocols which have been developed for *C. riparius* and *C. tentans* in Europe and North America (ASTM, 2010; OECD, 2010). It measures lethality and growth (over 5 days), and larval development, adult emergence and sex ratio following exposure to undiluted test sediments over a 10-day period (OECD, 2004a,b, 2010). The method is suitable for use with whole-sediment TIE (toxicity identification evaluation) procedures to assist in identifying the toxicants responsible for observed effects.

The life-cycle of *C. tepperi* can be divided into four distinct stages: (i) an egg stage, (ii) a larval stage consisting of four instars, (iii) a pupal stage, and (iv) an adult stage. Males are easily distinguished from females because they have large plumose antennae and a much thinner abdomen with visible genitalia (Fig. H.1). The male has paired genital claspers on the posterior tip of the abdomen. The adult female weighs about twice as much as the male, with ~30% of the female weight contributed by the eggs. After mating, adult females

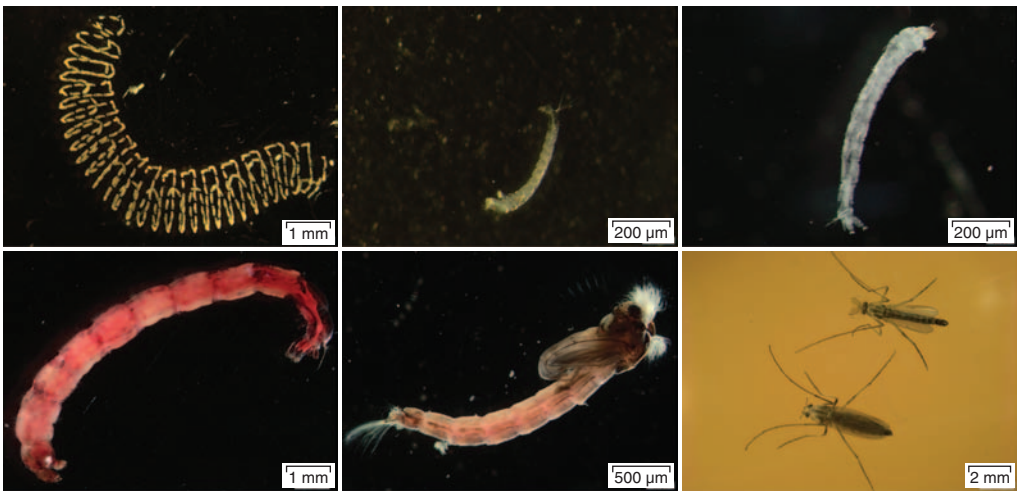

Figure H.1. Developmental stages of the chironomid *Chironomus tepperi*: (top left to right) egg case, 1st instar (Day 3), and 2nd instar (Day 5); (bottom left to right) instar, pupa and adults (male above, with brushy antennae, and larger female below) (photographs: Hai Doan).

oviposit a single transparent, gelatinous mass of eggs directly into the water. Egg cases contain a variable number of eggs from ~120 to 680 eggs per egg case which will hatch in 3–4 days at 21°C (Martin and Porter, 1978; Stevens, 1993; 1994). The four larval stages are followed by a black-coloured pupal stage (lasting ~3 days) and emergence to a terrestrial adult (imago) stage. Under optimal conditions, eggs will emerge as adults after ~14–15 days at 21°C. The adult stage lasts for 2–3 days, during which time the adults mate during flight and the females oviposit their egg cases.

After hatching from the egg stage, the chironomid larvae pass through four larval stages or instars before pupating and eventually emerging as an adult midge (typically, a life-cycle of several weeks to months). The larvae burrow into sediment and construct tubes that are open at both ends, and the larvae feed by extending their anterior ends outside the tubes to feed on detrital material on the sediment surface. The larvae ventilate their tubes with fresh water by undulations of the body, thereby facilitating gas exchange during times of low ambient oxygen. The undulations also draw in organic-rich surface-sediment particles for food and so the larvae may be directly exposed to sediment-bound contaminants. The larvae are prey for water birds and larger invertebrates and hence have the potential for trophic transfer of contaminants from sediments to higher trophic levels. These attributes of chironomids, along with their ease of handling and culturing in the laboratory, and their sensitivity to contaminated sediments, make them an appropriate species for sediment toxicity testing.

H.2 Method basis and summary

The 10-day chironomid bioassay described here was adapted from the methods described by ASTM (2010) and OECD (2004a,b, 2010). It measures, along with survival, the growth, emergence and sex-ratio following exposure of the first instar of *C. tepperi* to test sediments over a 10-day period. For the assay, ten 5-day old midge larvae (first instar) are added to 500 mL beakers containing ~140 g (wet weight) of 425 µm sieved sediment and 200 mL of moderately hard water, with 8 replicate beakers per sediment. After a 5-day incubation at

21°C (16-h light : 8-h dark) and before pupation, midge larvae are removed from 4 replicate beakers and their lengths are measured. Survival rates are also determined. The sediment exposure to midge larvae is continued for the other 4 replicate beakers to measure emergence and sex ratios. All data are analysed using a regression model to estimate the concentration that would cause X% reduction in the relevant endpoints (for example, EC10, EC50, etc.), or by using hypothesis testing to determine a no observed effect concentration (NOEC).

H.3 Method

H.3.1 Equipment

- Constant temperature chamber or temperature controlled room with 16-h light (intensity ~1000 lx; 10–20 µmol photons/s/m^2) : 8-h dark cycle, maintained at 21 ± 1°C.
- Test chambers: 500 mL glass beakers.
- Acrylic beaker lids.
- Air supply and tubing.
- Particle-size-matched control freshwater sediment.
- 20 L glass tanks and 1 L glass beakers (for culturing).
- Unbleached shredded tissue paper (for culturing).
- Mesh cover (250 µm) for culture tanks.
- Fish flake food (Tetra Min®, Tetra Phyll®).
- DO, pH and conductivity meters.
- Sieves: 425 µm mesh stainless-steel, and 2 mm mesh plastic.
- Sediment corer or stainless-steel spade or hand trowel to collect sediment.
- Portable cooler box with ice or cooler blocks.
- 10% buffered formalin.

H.3.2 Moderately hard water

Moderately hard freshwater, used for culturing and as overlying water in toxicity tests, is prepared according to the method recommended by USEPA (2002) (Table H.1).

H.3.3 Control and test sediments

Uncontaminated sediments with a range of properties can be collected from different freshwater locations for use as controls. As the detection of effects on survival, growth and emergence in test sediments is made by comparison to control sediments in the toxicity test, it is important that the control sediment not only has concentrations of contaminant that do not cause significant effects but also has similar physico-chemical parameters to the test sediment, including grain size and pore-water conductivity. For control sediments,

Table H.1. The preparation of moderately hard water, showing the salt concentrations required (mg/L) and the approximate final water quality (adapted from USEPA, 2002)

Reagent added (mg/L)				Approximate final water quality			
$NaHCO_3$	$CaSO_4.2H_2O$	$MgSO_4.7H_2O$	KCl	pH	Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	EC (µS/cm)
96	60	60	4	7.4–7.8	80–100	57–64	210–270

greater variation in physico-chemical properties can be achieved by mixing silty and sandy sediments with clean sand.

Control or test sediment, collected using a sediment corer, stainless-steel spade or hand trowel, is press-sieved (wearing gloves) on site through a 2 mm plastic mesh sieve to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in cooler boxes with ice or cooler blocks and returned to the laboratory and stored at 4°C. Sediment should not be frozen or allowed to dry out and should be used in tests as soon as possible and within 8 weeks of receipt. Before use, control and test sediments are held at room temperature (21 ± 1°C) for a minimum of 24 h to allow them to equilibrate to the test temperature.

H.3.4 Test organisms

Culturing chironomids

Shredded unbleached tissue paper is used as a substrate to culture *C. tepperi*. Tissue paper is cut into strips and soaked overnight in ethanol. After 4–5 washings with deionised water, the tissue paper is boiled and rewashed in deionised water. The dry shredded tissue paper placed in a 150 mL beaker produces enough substrate for one 20 L tank. Additional substrate can be frozen for later use.

Chironomus larvae are reared in 20 L glass tanks filled with moderately hard water to a depth of 15–20 cm. Water levels should be topped up as necessary to replace evaporative loss, and prevent desiccation. Water can be replaced if necessary. Gentle aeration should be provided. The larval rearing tanks are covered with fine mesh (250 µm) to prevent the escape of the emerging adults. The midge culture tanks should be maintained in a constant environment room at 21 ± 1°C with a photoperiod of 16-h light (intensity ~1000 lx or 10–20 µmol photons/s/m^2) : 8-h dark.

Five egg cases will provide a sufficient number of organisms to start a new culture tank. Egg cases are held at 21°C in a 1 L glass beaker containing ~800 mL of culture water (temperature change should not exceed 1°C per day). Food is not added until the embryos start to hatch (in ~3–4 days at 21°C) to reduce the risk of oxygen depletion. About 200 to 400 larvae are then placed into each 20 L culture tank. Crowding of larvae will reduce their growth.

Chironomus larvae are fed with a fish flake food (Tetra Min®, Tetra Phyll®) at ~250 mg per vessel per day, given as a suspension in water: 1.0 g of flake food is added to 20 mL of dilution water and blended to give a homogenous mix. This preparation is used at a rate of ~5 mL per vessel per day (shaking before use).

H.3.5 Toxicity test procedure

A summary of the test protocol is shown in Table H.2.

Larvae from aquaria-raised midges are used for the toxicity tests. Five days before testing, egg masses are collected from cultures, and placed in 1 L beakers (2 egg masses/beaker) with 800 mL of moderately hard water (210–270 µS/cm, pH 7.4–7.8, DO >60%) containing 7.5 g of artificial substrate (shredded tissue paper after washing and drying). Over the next 5 days, egg masses in these beakers are aerated continuously, fed twice with ground fish flakes (250 mg/vessel, on Days 3 and 4), and incubated under constant temperature conditions (21 ± 1°C) with a 16-h light : 8-h dark photoperiod using cool-white fluorescent lamps (10–20 µmol photons/s/m^2). Five-day-old larvae at first instar larval stage are needed for the tests. The cultures are considered suitable for use in toxicity tests if they have provided a constant supply of larvae, if the larvae are healthy and behave

Table H.2. Summary of test conditions for the 10-day midge *Chironomus tepperi* bioassays

Test type	Renewal of overlying water in test vessel
Test duration	Larval survival and growth: 5 days Emergence and sex ratios: 10–12 days
Temperature	21 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	1000 lx (10–20 µmol photons/s/m^2)
Photoperiod	16-h light: 8-h dark
Test chamber size	1 L
Test solution volume	140 g sediment plus 400 mL moderately hard water
Age of test organisms	1st instar
No. of organisms per replicate	10
No. of replicates per treatment	8 replicates (4 to be sacrificed on day 5, and the other 4 to be continued for the emergence test)
No. of organisms per treatment	40 + 40 (growth + emergence test)
Feeding regime	Fish flake suspension (Tetra Min®, Tetra Phyll®), 5 mL per vessel per day of feeding
Test chamber aeration	Aeration provided
Dilution water/overlying water	Moderately hard water (80–100 mg $CaCO_3$/L)
Dilution sediment (control sediment)	Usually sediment from reference site
No. of test concentrations	4–6
Endpoint	5 day survival and growth (length) 10–12 day emergence and sex ratios
Test acceptability criterion	≥80% survival in controls; reference toxicant LC50 within Cusum limits

normally, and if mortality has been ≤10%. A copper reference toxicity test (water-only exposures) is run at the start of whole-sediment bioassays using the same batch of larvae for 48 h to ensure their good health.

For the growth and survival bioassay, ten 5-day-old midge larvae are added to each replicate 500 mL beaker containing ~140 g (wet weight) of 425 µm sieved sediment and 200 mL of moderately hard water, with 4 replicates per treatment. Each beaker is maintained under the conditions described above. After 5 days, and before pupation, midge larvae from each replicate are removed by sieving the sediments and collecting live midge larvae. These larvae are fixed in 10% buffered formalin and processed for their length measurements using a stereomicroscope and image analysis software.

For assessing larval development (i.e. emergence as adults from sediment), similarly, ten 5-day-old midge larvae are added to beakers containing ~140 g (wet weight) of 425 µm sieved sediment and 200 mL of moderately hard water, with 4 replicates per treatment. The beakers are incubated for 10 days at 21°C (16-h light : 8-h dark) and the number of emerging adult *C. tepperi* is measured daily, and their genders are noted (males having large plumose antennae, much thinner abdomen and visible genitalia).

The pH and electrical conductivity are measured at the beginning and end of the bioassay; and dissolved oxygen and temperature in the test solutions are measured daily.

The 5-day growth and survival and 10-day larval development test endpoints are calculated using ToxCalc v5.0 (Tidepool Scientific Software), or a similar software. Point estimates for growth (IC50 and IC10) are calculated using linear interpolation (or similar, based on data assumptions) while point estimates for survival and larval development (EC50, EC10) are calculated using Spearman-Karber or Probit methods. The highest concentration of sample tested causing no significant toxicity (the NOEC) is determined using a hypothesis-based test (e.g. Dunnett's test). A reference toxicant test, using copper, is also carried out using *C. tepperi* larvae from the same batch of cultures used in the sediment bioassay.

H.3.6 Chemical and physico-chemical analyses

An additional replicate test container per treatment can be useful for monitoring chemical and physico-chemical parameters at the start of the test, throughout the test and at the test's termination. Physico-chemical parameters in the overlying water, including dissolved oxygen, pH, ammonia, electrical conductivity and temperature, are monitored to ensure that they remain within acceptable limits for quality control purposes.

Sediment and pore-water (0.45 µm filtered, acid-washed filtered) samples may also be taken from this beaker at test termination for toxicant analysis. Overlying water and pore-water samples for metals analysis are typically acidified with concentrated high purity nitric acid to a concentration of 0.2% and stored at 4°C until analysed. Samples for dissolved ammonia analyses may be frozen or preserved using sulfuric acid if analysis is not possible immediately, but analyses should ideally be undertaken as soon as possible.

H.3.7 Test acceptability and statistical analysis

Test conditions and physico-chemical parameters (dissolved oxygen, pH, conductivity and temperature) must remain within acceptable limits throughout the test.

Results of toxicity tests are reported in terms of the percentage survival, growth or emergence in test sediments relative to the same endpoint in the control sediment. For the test to be valid the following conditions apply:

- the mortality in the controls must not exceed 20% at the end of the test;
- emergence to adults should occur between 10 and 12 days after their insertion into the test vessels;
- the mean emergence in controls should be in the range 80–90%;
- at the end of the test, the dissolved oxygen concentration should be at least 60% of the air saturation value at the temperature used, and the pH of overlying water should be in the range 6–9 in all test vessels;
- the water temperature should not differ by more than 21 ± 1.0°C between vessels at any time during the test;
- A reference toxicant test should be run periodically to monitor the sensitivity of the bioassay.

Tests for significance between treatments and point estimate values (e.g. EC50) use ToxCalc for Microsoft Excel (Tidepool Scientific Software), or similar statistics software.

H.3.8 Reference toxicant tests

Due to the challenges of preparing reference sediments, a water-only reference toxicant test is recommended in preference to a whole-sediment test because it is simpler and takes less time. For the reference toxicant test, it is recommended that a 48-h survival test is conducted

using copper at concentrations in the range 16–512 μg/L and *C. tepperi* larvae from the same cultures as are used in the whole-sediment toxicity testing, to check that the cultures are of appropriate sensitivity. Dissolved copper concentrations should be measured at least three times throughout the tests to provide time-averaged concentrations for effects analysis.

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Appendix I: Protocol for whole-sediment acute and sub-lethal toxicity tests using the freshwater pond snail *Physa acuta*

Anu Kumar and Stuart L. Simpson

I.1 Introduction

This appendix describes a method for detection of any effects of sediment contaminants on the egg-production capacity of the freshwater snail *Physa acuta* (Mollusca, Gastropoda, Physidae) (Fig. I.1). The method was originally developed for the *in situ* monitoring of toxicity effects of drainage or stormwater or wastewater discharge, but it can equally be applied to sediments under field or laboratory conditions. The species is widely distributed, relatively easy to culture under controlled laboratory conditions, sensitive to a range of chemical contaminants and thus appropriate for toxicity testing (Kefford *et al*., 2004; Evans-White and Lamberti, 2009; Hossain and Aditya, 2013; Seeland *et al*., 2013). The method represents a sensitive and robust approach for undertaking whole-sediment toxicity tests on freshwater sediments with acute and sub-lethal, chronic test endpoints.

Physa acuta is an invasive species that is now widespread in freshwaters worldwide (Albrecht *et al*., 2009; Guo *et al*., 2009). Snail (gastropod) species in general are widely distributed and have relatively high abundances in many freshwater environments. Due to their abundance and size, they play an important role in many food webs, both as decomposers and as consumers; most are herbivores and very few are carnivores. Many species tolerate wide variations in electrical conductivity and temperature, and some are suitably sensitive to contaminants for use in ecotoxicology testing programs (Duft *et al*., 2003a,b; Ma *et al*., 2010). Many are deposit feeders, ingesting sediment particles and organic detritus, algae and bacteria in the sediment or other organic carbon-rich substrates, and these species are expected to be highly exposed to sediment-associated contaminants as a consequence. Snails are prey for water birds and hence there is the potential for trophic transfer of contaminants from sediments to higher trophic levels. These attributes of snails, along with their ease of handling and culturing in the laboratory and their sensitivity to contaminated sediments, make them appropriate species for sediment toxicity testing.

The species *Physa acuta* is a small, left-handed or sinistral, air-breathing freshwater snail that lives in freshwater rivers, streams, lakes, ponds and swamps. It eats dead plant and

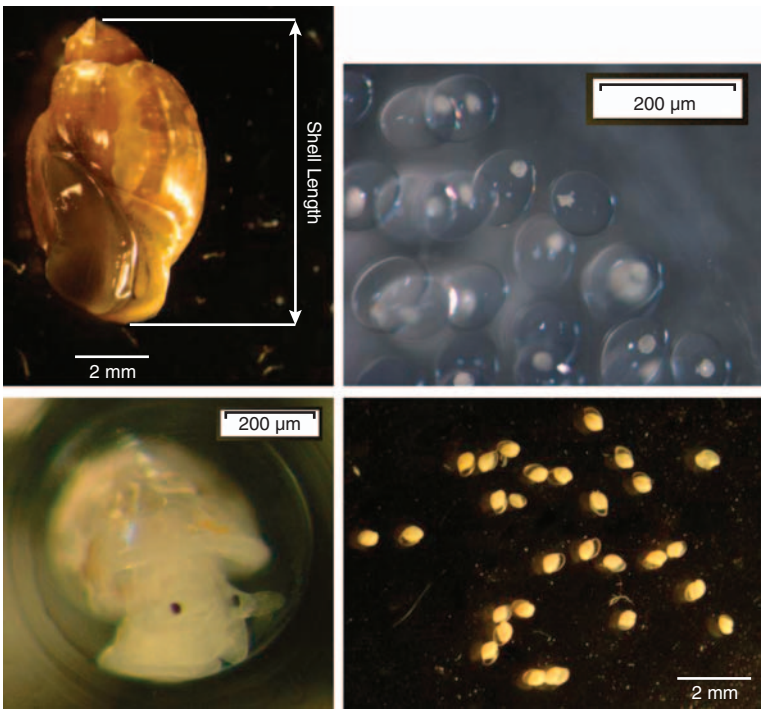

Figure I.1. The snail *Physa acuta*: (top left) adult; (top right) egg sacs collected from adult snails after a one-week deployment; (lower left) normal larval development; (lower right) juvenile snails (photographs: Hai Doan).

animal matter and various other detritus. These snails mature in 6–8 weeks, male function arriving slightly before female function, each adult laying 50–100 eggs every 2–3 days thereafter for up to a year. Being hermaphrodites, any two of these snails can breed. During breeding, both individuals exchange genetic material, and subsequently both will lay eggs masses comprising 50–400 eggs. The species is also capable of asexual reproduction, self-fertilising in a procedure that is neither cloning nor parthenogenesis. Each egg, even when self-fertilised, will be genetically unique based on the coming together of the two halves. In addition to its ability to self-fertilise, *P. acuta* can store genetic material. A single encounter with another pond snail could provide enough genetic raw material for many clutches of eggs. This species successfully co-exists with other alien gastropods, e.g. the mud snail *Potamopyrgus antipodarum*, in many streams, lakes and ponds in Australia.

The toxicity test protocol for *P. acuta* measures the effects of sediment quality on egg production, larval hatching success and growth of snails following exposure to undiluted test sediments over a 28-day period. While the method describes the procedures for laboratory exposures, *in situ* deployment may be undertaken where the snails are caged on or above the sediment, providing a means of assessing the combined effects of water and sediment quality. *Physa acuta* is suitable for testing for the effects of contaminants in a full spectrum of sediment types (sand to silt, high to low organic carbon), and in environments where the waters have a wide range of electrical conductivities and temperatures.

The test procedure describes a relatively rapid, standardised lethal and sub-lethal toxicity test that provides an indication of the potential toxicity of freshwater sediment samples to local biota. The egg production, larval hatching success and growth endpoints indicate the potential for long-term effects at the individual and population level.

The multiple test endpoints (lethal and sub-lethal) make it a very useful whole-sediment test method for sediment quality assessments.

I.2 Method basis and summary

For the assay, 10 adult snails, 10–12 mm in size, are placed either (a) on the sediments in 1 L beakers, or (b) in cages *in situ* that can be either above the sediment or pushed into the sediment allowing direct contact. The cages are covered with net of mesh size approximately 200 μm.

Four independent replicates are used per laboratory test or at each site when field deployed. When deploying snails directly onto sediments, the substrates should be sufficiently consolidated to allow the snails to maintain themselves at the surface; that is, without sinking and potentially suffocating from a lack of oxygen. This is not a problem when snails are caged above the sediments to assess exposure in the water phase.

For the egg-production test, 10 snails of similar shell size are held for 7 days in test chambers. During this time, the snails lay 'egg masses' on the walls of the test chambers. An 'egg mass' refers to a discrete batch of eggs that is surrounded by a gelatinous coating. Each egg lies in its own capsule within the gelatinous mass. At the end of the 7-day test period, the number of eggs produced at each site is counted. These values are compared between sediments or sites, and with values obtained in previous tests. Typically ~4–6 weeks of monitoring can be conducted in a given season. The egg masses collected from each test chamber are transferred to new containers in the laboratory and maintained in water and sediment from the field sites for another 21 days to assess their hatching success and the growth of juvenile snails. The sizes of the hatched juvenile snails are measured using image analysis software and a stereoscopic microscope. Survival rates of adults are determined, and biomarker responses may also be investigated as additional endpoints.

At the end of tests, all the laboratory data are analysed using a regression model to estimate the concentration that would cause X% reduction (for example, EC10 or EC50) in the relevant endpoints when exposed to the known contaminants under laboratory conditions. For field assessment, data from various different endpoints are compared with the reference site and laboratory controls using one way analysis of variance (ANOVA) to identify the contaminated sites.

I.3 Method

I.3.1 Equipment and supplies

- Constant temperature chamber or temperature controlled room with 16-h light (intensity ~1000 lx; 10–20 μmol photons/s/m^2) : 8-h dark cycle, maintained at 21 ± 1°C.
- Exposure cages (Perspex® tubes 15 cm long × 10 cm diameter, with 0.5 cm wall thickness).
- Nylon mesh: 200 μm mesh.
- Test chambers (laboratory): 1 L glass beakers.
- Acrylic beaker lids.
- Air supply and tubing.
- Diluent/control field water or laboratory water with specified properties (pH, conductivity, hardness).
- Particle-size-matched control freshwater sediment.
- 20 L glass tanks (for culturing).

Table I.1. Reagent grade chemicals and commercial salts for preparing 200 L of artificial freshwater

Reagents added (g)						Water quality	
$MgSO_4 \cdot 7H_2O$	$NaHCO_3$	$CaCl.2H_2O$	NaCl	KCl	$CaSO_4 \cdot 2H_2O$	pH	EC (µS/cm)
15	19.2	3	80	6	12	6.8–7.5	1200–1500

- Cultured snails are fed commercial algae wafers (Hikari®) *ad libitum*.
- For bioassays, fish flake food (Tetra Min®, Tetra Phyll®) at 5 mg/snail/day.
- Dissolved oxygen, pH and conductivity meters.
- Stainless-steel spade or hand trowel to collect control sediment.
- Portable cooler box with ice or cooler blocks.

I.3.2 Synthetic water to culture snails

The standard synthetic water is prepared with deionised water and reagent grade chemicals and commercial salts as described in (Table I.1).

I.3.3 Control sediment

Uncontaminated sediments with a range of properties can be collected from different freshwater locations for use as controls. As the toxicity test is done to detect effects on survival, growth and hatching success in test sediments in comparison to control sediments, it is important that the control sediment not only has concentrations of contaminants that do not cause significant effects but also has similar physico-chemical parameters to the test sediment, including grain size and pore-water conductivity.

Control sediments can be collected using a stainless-steel spade or hand trowel. Sediments are press sieved (wearing gloves) on-site through a 2 mm plastic mesh sieve to remove large pieces of organic matter and organisms. Sieved sediment is collected in plastic bags and placed in portable cooler boxes with ice or cooler blocks, and returned to the laboratory and stored at 4°C for a maximum of 2 months. Before use in tests, control sediments should be held at room temperature for a minimum of 24 h to allow them to equilibrate to the test temperature. For control sediments, a range of physico-chemical properties can be achieved by mixing silty and sandy sediments with clean sand.

I.3.4 Test sediment

Test sediments should be collected and stored in containers made of inert materials to prevent contamination. Sediment should be chilled when collected, shipped on ice and stored in the dark in a refrigerator at 4°C. It should not be frozen nor allowed to dry out and should be used in tests as soon as possible and within 8 weeks of receipt. Before use, test sediment is held at room temperature (e.g. 21 ± 1°C) for a minimum of 24 h to allow it to equilibrate to the test temperature.

I.3.5 Test organisms

Culturing of snails

Physa acuta is a common pulmonate snail mostly found in streams, ponds, lakes throughout Australia. Field-collected breeding stocks can be maintained for at least 3–4 months under controlled laboratory conditions before testing. From cultures maintained in the laboratory, breeding stocks of adult *P. acuta* are placed into 2 L beakers for collection of egg masses for testing (4 individuals per beaker; 8 replicate beakers). Water is renewed

once per week and egg masses are collected 24 h after each water renewal. Snails and egg masses are maintained in artificial freshwater at 21 ± 1°C and 16-h light : 8-h dark photoperiod. Cultured snails are fed commercial algae wafers (Hikari®) *ad libitum*.

Snails used for field testing are also obtained from laboratory culture. Careful handling is required to avoid any damage to their shells before deployment of the snails in the field.

Supplementation of laboratory stocks with new wild snails from pristine sites should be programmed for 5 year intervals to minimise the risk of inbreeding that could potentially affect snail sensitivity. Ensuring adequate health and numbers of snails for toxicity monitoring requires close attention to the husbandry of *P. acuta*. Adequacy of snail stocks should be assessed at least 2 months before commencement of testing each wet season. This allows sufficient time for set up of new cultures if numbers of young adults are likely to be insufficient for the start of wet season testing. All test animals must be free of overt signs of disease or shell damage. Snails used in a toxicity test must not be used in subsequent tests.

Embryonic development stage

Physa acuta development includes four main embryonic stages – morula, trochophore, veliger, hippo – and the formation of the shell. At 21 ± 1°C, the time period for hatching is ~12–13 days (Table I.2).

I.3.6 Toxicity test procedure

A summary of the test protocol is shown in Table I.3.

The cages in which the snails are deployed in field tests are constructed from Perspex® tubes 15 cm in length and 10 cm in diameter (with a wall thickness of 0.5 cm) (Fig. I.2). For oxygen supply, gauze with mesh size 200 μm is fixed at the two ends of each cage, allowing

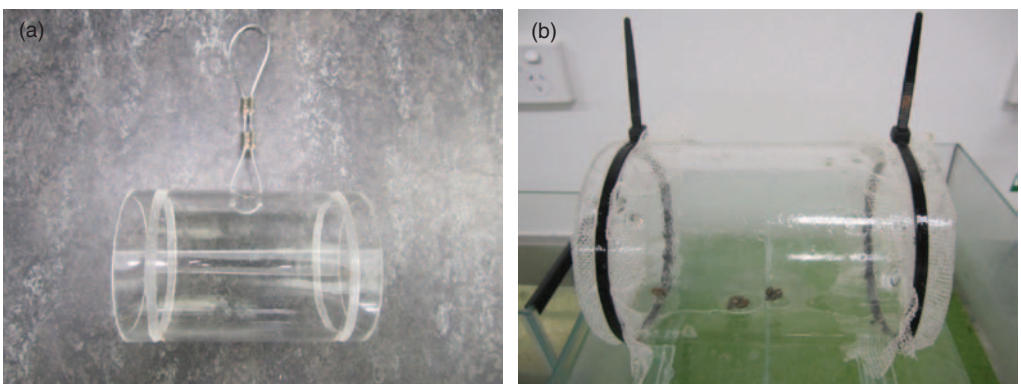

Figure I.2. Egg-laying chambers showing mesh screens held in place by circlips (photographs: Hai Doan).

Table I.2. Growth stages of *Physa acuta*

Development stage	Time period
Morula	1st day
Trocophore	4th day
Veliger	6th day
Hippo	8th day
Embryo hatching	12th day

adequate exchange of water. In the laboratory, the cages are deployed on top of 200–300 g of sediments, such that sediment may penetrate the mesh at the bottom but the snails cannot bury themselves deeply in it. The sediment and cages are held in containers large enough to allow adequate water circulation: 2 L beakers are considered a minimum size as containers.

I.3.7 Snail selection and field set-up

Snails with a size range of 10–12 mm shell length are taken from laboratory stocks early on the morning of Day 1 of the test, and sorted into groups of similar size for deployment. Shell length is taken as the distance between the apical tip and the shoulder (the longest dimension of the shell) (Fig. I.1). Snail selection is assisted by collecting from cohorts of known age. For each sediment tested, 40 snails are required. Careful handling of snails is necessary to prevent damage to shells during collection and measurement. A small number of extra snails (5–10) are required for replacement of dead or ailing individuals that may be observed before commencing tests. No snails outside of the size range 10–12 mm should be used, and where the groups of 10 snails have a range of sizes then the size classes must be distributed evenly among and between the duplicates of the different treatments.

Snails are put into the cages and the open ends are then covered by mesh (200 μm) which is secured to retain the snails. The groups of four egg-laying chambers per site with their enclosed snails are carefully transported to the field in containers holding water from the laboratory snail stock-tank.

The test is deemed to have commenced when all egg-laying cages have been placed in the test containers, the test-container lids are locked in place and the test containers are positioned appropriately. Placement of all cages for the field test must be completed within 1 hour or less, to ensure equal exposure time and similar water and sediment conditions for all cages, thereby minimising the chance that a longer or shorter exposure period will have led to the numbers of eggs differing between sediments or sites.

A field test runs for 7 days. During this test period, physico-chemical (laboratory and field) and hydrological (field) data must be collected for all test sites. It is important for proper interpretation of toxicity monitoring data that water quality data are available for each testing period. Particularly during field tests, the light exposure, temperature and water exchange rate can vary considerably. Temperature and electrical conductivity should be monitored several times per week in all waters during tests. Continuous monitoring of water temperature, electrical conductivity, pH and turbidity can be undertaken at each field site.

After 7 days of exposure, survival of adults is determined and counts are made of eggs and egg masses deposited on the inner surfaces of the cages. Each cage must be transported carefully from the test site in the laboratory or the field to a new container holding a sufficient volume of its respective treatment water to cover the cage. Considerable care must be taken in dislodging snails from the inner walls of the cages to prevent damage to the egg masses. At this stage, egg masses that contain no viable embryos are included in the counts. Otherwise, unusual and anomalous egg masses and embryos (e.g. absence of cell capsules, twin embryos in a single cell, damaged egg masses) are noted.

The field exposures are continued under laboratory conditions for another 21 days. For these laboratory exposures, surface water and sediment samples are collected from the field sites. Overlying water from the field site or laboratory water prepared with the desired properties (e.g. pH, conductivity and hardness) should be used and water exchanges should be made twice per week unless a higher rate of renewal is desired. The water within the cages should be aerated gently and continuously, and the cages should be incubated under constant temperature conditions (21 ± 1°C) with a 16-h light : 8-h dark photoperiod using

cool-white fluorescent lamps (10–20 µmol photons/s/m^2). If necessary, ground fish flakes can be added as a food source twice weekly (e.g. the day before water renewals).

For the test to be valid the following conditions must apply:

- the mortality in the laboratory controls and reference site samples must not exceed 20% at the end of the test;
- egg masses for controls should be ≥9;
- the mean growth for controls should be ≥1000 µm (1 mm);
- during the tests, the dissolved oxygen concentration should be at least 60% of the air saturation value (ASV) at the temperature used, and the pH of overlying water should be in the range 6–9 in all test vessels;
- the water temperature should remain within the desired range, i.e. 21 ± 1°C in the laboratory during the test.

Table I.3. Summary of test conditions for the 7-day survival and egg production, and 10-day larval hatching success and growth of the snail *Physa acuta*

Test type	Renewal of overlying water in test vessel
Test duration	Survival and egg production: 7 days Larval hatching success and juvenile snail growth: 21 days (following 1st stage)
Temperature	21 ± 1°C
Light quality	cool-white fluorescent tube lighting
Light intensity	1000 lx or 10–20 µmol photons/s/m^2
Photoperiod	16-h light : 8-h dark
Test chamber size	Exposure cages (Perspex® tubes 15 cm long × 10 cm diameter, with 0.5 cm wall thickness) placed within 2 L beakers (in laboratory) or secured in the field environment
Test solution volume	Laboratory: 200–300 g sediment and ≥800 mL water
Age of test organisms	10–12 mm shell length (cohorts of known-age)
No. of organisms per replicate	10
No. of replicates per treatment	4 replicates
No. of organisms per treatment	40
Feeding regime	Fish flake suspension (Tetra Min®, Tetra Phyll®) *ad libitum*
Test chamber aeration	Aeration provided to water around exposure chamber in laboratory
Dilution water/overlying water	Field site water or laboratory water of specified properties (e.g. pH, conductivity, hardness)
Dilution sediment (control sediment)	Usually sediment from reference site
Test concentrations	4–6 (when using spiked sediments or dilution series)
Endpoints	7-day survival and egg production 21-day larval hatching success and juvenile snail growth (length)
Test acceptability criterion	≥80% survival in controls; Reference toxicant LC50 within Cusum limits

Results of toxicity tests are reported in terms of the percentage of each endpoint (survival, egg production, larval hatching success and growth) in test sediments relative to the same endpoint in the control sediment. Toxicity is detected when the result for the test sediment endpoint is <85% of the control (based on 2 standard deviations of control data, $n = 30$) and significantly less (t-test, $P < 0.05$) than the same endpoint observed in the control. A reference toxicant test should be undertaken periodically to monitor the sensitivity of the bioassay.

I.3.8 Chemical and physico-chemical analyses

An additional replicate test container per treatment can be useful for monitoring chemical and physico-chemical parameters at the start of the test, throughout the test and at test termination. Physico-chemical parameters in the overlying water, including dissolved oxygen, pH, ammonia, electrical conductivity and temperature, are monitored to ensure that they remain within acceptable limits for quality control purposes.

Sediment and pore-water (0.45 µm filtered, acid-washed filtered) samples may also be taken from this beaker at test termination for toxicant analysis. Overlying water and pore-water samples for metals analysis are typically acidified with concentrated high purity nitric acid to a concentration of 0.2% and stored at 4°C until analysed. Samples for dissolved ammonia analyses may be preserved with sulfuric acid (below pH 2) and stored cold or frozen if analysis is not possible immediately, but analyses should ideally be undertaken as soon as possible. The freeze-dried sediment samples can also be used for the analyses of organics including pesticides, surfactants and pharmaceuticals.

I.3.9 Test acceptability and statistical analysis

Test conditions and physico-chemical parameters (dissolved oxygen, pH, conductivity and temperature) must remain within acceptable limits throughout the test.

Results of toxicity tests are reported in terms of the percentage survival, growth or emergence in test sediments relative to same endpoint in the control sediment. Toxicity is detected when the result for the test sediment endpoint is <85% of the control (based on 2 standard deviations of control data, $n = 30$) and significantly less (t-test, $P < 0.05$) than the same endpoint observed in the control.

I.3.10 Reference toxicant tests

Due to the challenges of preparing reference sediments, a water-only reference toxicant test is recommended in preference to a whole-sediment test because it is easier and takes less time. For the reference toxicant test, it is recommended that a 48-h survival test be conducted using copper at concentrations in the range 10–500 µg/L and *P. acuta* from the same cultures as are used in the whole-sediment toxicity testing, to check that the cultures are of appropriate sensitivity. Dissolved copper concentrations should be measured at least three times throughout the tests to provide time-averaged concentrations for effects analysis.

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Appendix J: Protocol for whole-sediment bivalve biomarker assays using *Tellina deltoidalis* and *Anadara trapezia*

Anne M. Taylor

J.1 Introduction

This guide describes methods for three biomarker assays for sub-lethal toxicity to bivalves exposed to metal-contaminated sediments (Taylor and Maher, 2010, 2012a, 2014). They comprise lipid peroxidation, lysosomal stability and micronuclei occurrence, and they respectively consider enzymatic, cellular and genotoxic effects. The two test bivalves are *Tellina deltoidalis*, a small benthic deposit-feeding marine bivalve that is best suited to 28-day exposures in laboratory microcosms with no supplementary feeding, and *Anadara trapezia*, a benthic sediment-dwelling filter-feeding marine bivalve that is better suited to longer-term 60-day exposures, with supplementary feeding, to ensure tissue contaminants reach a steady-state. *Anadara trapezia* is also suitable for *in situ* field bioassays in cages (ASTM, 2013).

The use of bivalves for biomarker tests is common worldwide and recommended by many regulatory bodies, e.g. USEPA (2000) and ASTM (2010). Bivalves are representative of sediment infauna, and are in direct contact with contaminants, through filter-feeding from the water column and, in the case of *T. deltoidalis*, also through deposit-feeding on sediment particles. Their burrowing activities also facilitate resuspension and mobilisation of contaminants to pore waters and surface waters. *Tellina deltoidalis* and *A. trapezia* satisfy the general requirements for effective biomonitors: they are hardy, accumulate metals and other toxicants, have sufficient tissue for analysis, are relatively abundant around Australia, and are suitable for laboratory studies.

Current developments in ecotoxicological assessment have made acceptable the use of sub-lethal endpoints for determining guideline concentrations for toxicant exposures. Biomarkers of exposure and effects have been progressively developed and refined for a range of toxicants and aquatic species for application in environmental assessment (see Chapter 6). Biomarker measurements produce information on effects that cannot be obtained through measurements of contaminants in environmental media or as tissue concentrations. Biomarkers have the potential to provide evidence that organisms have been

exposed to contaminants at levels that exceed their capacities to detoxify and repair themselves, and to establish links between toxicant exposure and ecologically relevant effects.

J.2 Methods basis and summary

The tests described below determine biomarker responses of deposit- and filter-feeding benthic bivalves, to metals in estuarine or marine sediments. The tests can be conducted with sediments that are spiked with the metals of interest at known concentrations, or with field-contaminated sediments in microcosms. Tests should include a minimum of 3 replicates per exposure treatment and a control of clean sediment of similar matrix, with 10–15 individuals per treatment. The organisms are young adult *T. deltoidalis* 15–25 mm long, and *A. trapezia* 30–50 mm long, obtained from uncontaminated estuaries. Each test with *T. deltoidalis* uses 50 g of wet sediment (with a minimum of 20% <63 μm fraction) per 1 g wet tissue mass, to ensure the sediment depth is sufficient for burrowing (approx. 60 mm minimum). For tests with *A. trapezia*, a sediment depth of 1.5 times the maximum shell length is required for burrowing. The dose–response is measured after 28 days for *T. deltoidalis* and 60 days for *A. trapezia*, and is compared to the response in individuals of the same species exposed to control sediments. An additional endpoint can include a condition index calculated as a ratio of the total tissue mass to shell mass.

J.2.1 Lipid peroxidation

Increased lipid peroxidation is a widely reported consequence of the excess oxyradical production in marine bivalves which can occur in response to metal exposure (Almeida *et al*., 2004; Company *et al*., 2004; Taylor and Maher, 2012b, 2013). Broadly defined, it involves the oxidative deterioration of polyunsaturated fatty acids, which results in the production of highly reactive and unstable lipid radicals and a variety of lipid degradation products. The most abundant lipid degradation product is malondialdehyde (MDA), which can alter the structure of cell membranes (Viarengo, 1989). The process of lipid peroxidation destabilises cell membranes which can lead to the leaking of the lysosomal contents into the cytoplasm and produces cytotoxic compounds which can damage DNA (Winston *et al*., 1996; Kehrer, 2000).

The thiobarbituric acid reactive substances (TBARS) assay is a well-established method used to measure lipid peroxidation by measuring the MDA concentration in tissue lysates. The MDA end product of lipid peroxidation forms a 1:2 adduct with TBARS and produces a colour reaction that can be read spectrophotometrically at 532 nm and compared to an MDA standard curve. MDA values should be normalised to the protein content of the samples and MDA expressed per mg of protein content.

The concentration of protein in each of the tissue lysates can be measured using the Bradford assay, a simple and rapid colorimetric method (Bradford, 1976). It involves the binding of Coomassie Brilliant Blue G-250 dye to proteins, which converts the unstable red cationic form (A_{max} = 470 nm) to a stable anionic blue form (A_{max} = 595 nm). The blue protein-dye is detected at 595 nm.

J.2.2 Lysosomal stability assay

Lysosomes are intracellular organelles that contain acid hydrolases for the digestion of cellular waste, including excess or damaged organelles, food particles, viruses and bacteria. The lysosome interior is more acidic (pH≈4.8) than the cytosol (pH≈7.2) and is enclosed in a single membrane which stabilises the low pH by pumping protons from the cytosol via

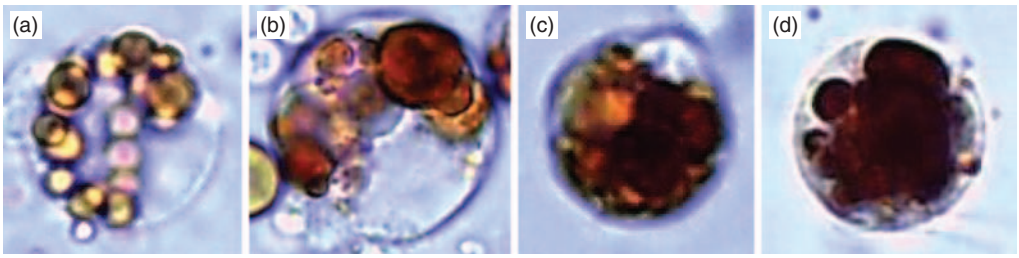

Figure J.1. Hepatopancreas cells: (a,c) *Anadara trapezia*; (b,d) *Tellina deltoidalis*; (a,b) with dye retained in the lysosomes, i.e. stable; (c,d) with dye present in the cytosol, i.e. unstable (magnification 400×) (photographs: Anne Taylor).

proton pumps and ion channels. Lysosomes also play an important role in sequestration and detoxification of metals and other contaminants (Viarengo, 1989). Disruption of the proton pump, by contaminants or by-products of oxidative damage such as from lipid peroxidation, can disrupt the lysosomal membrane causing the acidic contents to leak into the cell cytosol leading to cell death (Lowe and Fossato, 2000). Lysosomal perturbations have been widely used as early indicators of adverse effects from exposure to pollutants (Galloway *et al*., 2004; Moore *et al*., 2004) and are considered to be an effective prognostic biomarker, because lysosomal destabilisation frequently precedes higher order cell and tissue pathologies (Kirchin *et al*., 1992; Köhler *et al*., 2002; Allen and Moore, 2004; Moore *et al*., 2006). The lysosomal membrane stability assay has been adopted as a prognostic tool by international assessment agencies including the United Nations Environment Programme (UNEP/RAMOGE, 1999) as part of the first tier of techniques for assessing harmful impact in the Mediterranean Pollution programme (MEDPOL Phase IV). It is also recommended as a biomarker to be included in the Convention for the Protection of the Marine Environment of the North-East Atlantic Coordinated Environmental Monitoring Programme (OSPAR, 2008).

The neutral red retention test on living cells has been developed for several marine molluscs (Lowe and Pipe, 1994; Cheung *et al*., 1998; Ringwood *et al*., 1998; Nicholson, 1999) and is a recognised method for measuring lysosomal membrane stability. The assay has been used successfully with mollusc haemocytes (Lowe *et al*., 1995a; Nicholson, 2003) and hepatic cells (Lowe *et al*., 1995a; Ringwood *et al*., 2002) with similar results between them. Hepatic cell preparations generally provide more cells than haemocytes do, and are the only suitable option for small organisms such as *T. deltoidalis* and those with red haemolymph such as *A. trapezia*. The protocol, based on a method developed for the oyster *Crassostrea virginica* by Ringwood *et al.* (2003), details the isolation of cells from hepatopancreas tissue and incubation in neutral red. Living cells incubated in the lipophilic neutral red dye accumulate it in their lysosomes. In healthy cells it is retained in the lysosomes; in cells with damaged lysosomal membranes it leaks out into the cell cytoplasm, resulting in cell death (Lowe *et al*., 1995b) (Fig. J.1).

J.2.3 Micronuclei assay

Micronuclei are small intracytoplasmic masses of chromatin resulting from chromosomal breakage or aneuploidy during cell division (Bolognesi *et al*., 2004). They resemble the main nucleus and are easily observed in interphasic cells (Scarpato *et al*., 1990). The micronucleus assay is one of the most promising techniques for identifying genetic alterations in organisms exposed to toxicants (Bolognesi *et al*., 2004). As an index of chromosomal

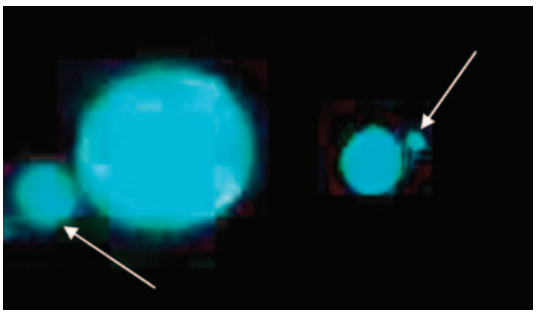

Figure J.2. Examples of DAPI-stained nuclei with associated micronuclei from gill tissue of *Anadara trapezia* with arrows indicating micronuclei (magnification 1000×) (photograph: Anne Taylor).

damage the micronuclei test is based on the enumeration of downstream aberrations after DNA damage and gives a time-integrated response to toxic exposure. The test is fast and sensitive and has proved suitable for application to aquatic invertebrates; it is simpler and more rapid than other measurements of chromosomal aberration (Burgeot *et al.*, 1996). It has been used in bivalves to examine the genotoxicity of a range of chemicals including metals (Scarpato *et al.*, 1990; Williams and Metcalfe, 1992; Burgeot *et al.*, 1996; Bolognesi *et al.*, 2004). The assay uses DAPI (4',6-diamidino-2'-phenylindole dihydrochloride), a fluorescent dye specific for nucleic material, to stain the nuclei. Micronuclei are defined as small round structures less than one-third the diameter and in the same optical plane as the main nucleus, with a boundary distinct from the nuclear boundary (Fig. J.2).

J.3 Exposure procedures

J.3.1 Equipment

- Temperature and light controlled room or chamber 22 ± 1°C, with a 14-h light (3.5 µmol photons/s/m^2) : 10-h dark cycle.
- Test chambers: glass or polystyrene containers, minimum size 1 L for *T. deltoidalis*, 10 L for *A. trapezia*.
- Air pump, air stones, air flow control valves and tubing.
- Clean seawater.
- Dissolved oxygen meter, pH meter, conductivity meter or seawater refractometer.
- 2 mm and 1 cm mesh stainless-steel sieves.
- Stainless-steel shovel or trowel for *T. deltoidalis* and sediment collection.
- Portable cooler boxes and plastic buckets with lids for transportation of field-collected organisms and sediments.
- Glass or polystyrene holding aquariums with clean sediments and salinity-matched seawater for organism acclimatisation before experimentation.

J.3.2 Materials

Sediment

Control sediments can be collected from the same site as test organisms or from an uncontaminated site with the same geochemical sediment properties, to suit organisms' feeding

and burrowing requirements. Sediments should be collected with a stainless-steel shovel or trowel, and press-sieved through 2 mm stainless-steel mesh to remove large pieces of organic material and organisms. Sieved sediments are placed in plastic buckets with lids, and sealed with tape for transport. Sediments can be stored at 4°C for up to 2 months. Test sediments are collected, transported and stored in the same way as control sediments. Before tests begin, sub-samples of sediments should be analysed to measure their metal concentrations, to establish metal exposures. Moisture content, salinity, pH and sediment grain size should also be measured to establish the physico-chemical properties of control and test sediments. Properties of control and test sediments should be closely matched to ensure the results for contaminant dose and effects are not influenced by physico-chemical factors rather than the contaminants of interest.

Water

Seawater for use in the tests should be collected from uncontaminated coastal waters. Its salinity can be adjusted with deionised water to match the salinity of the water where the test organisms were collected, usually 30 ± 2‰.

Bivalves

Tellina deltoidalis is collected from uncontaminated estuarine sediments at low tide, using a stainless-steel shovel to lift out the top 10–20 cm layer of sediment which is then sifted through a 1 cm mesh stainless-steel sieve to collect bivalves of 15–25 mm shell length.

Anadara trapezia can be found in estuaries in and around seagrass beds, generally at wading depth. These filter-feeding bivalves live at the sediment surface and can be collected by hand, by gently sweeping gloved fingers through the surface sediments.

For transportation, the collected organisms are placed in a portable cooler box containing sediment and water from the collection site. An aquarium air pump should be used to aerate overlying water during transportation and maintain ambient temperature. Collected bivalves should be kept in aquaria for up to 2 weeks to acclimatise before experimentation. Holding tanks should be prepared, with control sediments 10 cm deep and with water of the same salinity as that at the bivalve collection site. Inline control valves on air hoses enable the overlying waters in the aquaria to be aerated to achieve ≥85% oxygen saturation without disturbing sediments. Water temperature should be 22 ± 1°C, and the photoperiod should be 14-h light : 10-h dark. If ambient water temperature at the time of collection is 10 ± 5°C cooler or warmer than 22 ± 1°C, the water temperature of the holding tanks must be adjusted gradually at 2 ± 0.5°C per day until the experimental temperature is reached. A 3-day feeding/half water change cycle should be maintained during the acclimatisation period, using a suitable supplementary food such as the unicellular green algae *Nannochloropsis* preparation (Nanno 3600, Instant Algae®, USA).

J.3.3 Methods

Exposure test protocols for *T. deltoidalis* and *A. trapezia* are summarised in Table J.1. The required mass of control or test sediment should be weighed into a minimum of 3 test chambers per treatment, 48 h before the start of the experiment. Sediments should be covered with cling film and placed in a temperature controlled area. After 24 h of sediment temperature equilibration, seawater is added by pouring it down a baffle and removing the cling film, taking care to minimise disturbance to the sediments. Aeration is introduced to surface waters and the airflow is adjusted to ensure sediments are not disturbed. The test system should be allowed a further 24 h to equilibrate before organisms are introduced.

Laboratory test initiation

To establish the background metal concentrations and biomarker responses of the test organism population, it is recommended that a sub-set of the population from the holding tanks, 6–10 individuals, be measured before starting the test exposures.

The required number of organisms should be placed in the test chambers on the sediment surface by hand, and monitored throughout the day to ensure normal burrowing activity.

Laboratory test maintenance

Test chambers should be checked, morning and evening, for air flow and to observe organism behaviour and health. Any dead organisms should be removed.

Half (50%) of the overlying water in *A. trapezia* test chambers should be replaced every 3 days, and these organisms should be fed with a suitable algal preparation at 1% (v/w) of total organism wet tissue mass immediately after water is changed.

Bivalve depuration

At the end of the exposure period, all organisms are removed from sediments and placed for 24 h in static chambers containing seawater, as used in test exposures, for depuration of gut contents, to ensure tissue metal concentration results are not influenced by sediment particles. Depuration chambers are maintained at the same temperature and light conditions as were used in the tests.

Table J.1. Summary of exposure test conditions for biomarker sediment toxicity tests

Species	*T. deltoidalis,* 28-day exposure	*A. trapezia,* 60-day exposure
Test type	Static non-renewal	Non- static renewal
Test duration	28 days	60 days
Temperature	22 ± 1°C	22 ± 1°C
Salinity	30 ± 2‰ or as required	30 ± 2‰ or as required
pH	7.8–8.2	7.8–8.2
Light intensity	3.5 µmol photons/s/m^2	3.5 µmol photons/s/m^2
Photoperiod	14-h light : 10-h dark	14-h light : 10-h dark
Aeration	Maintain ≥85% oxygen saturation without disturbing sediments	Maintain ≥85% oxygen saturation without disturbing sediments
Test chamber	1–3 L glass or polystyrene containers or aquaria	10–12 L glass or polystyrene containers or aquaria
Sediment mass	50 g of wet sediment (with a minimum of 20% <63 µm fraction) per 1 g wet tissue mass	1000 g of wet sediment (with a minimum of 20% <63 µm fraction)
Overlying water volume	Equal volume to the sediment mass used	8–10 L
Organism size	15–25 mm	30–50 mm
Organisms per test chamber	10–15	10–15
Replicate test chambers	3 minimum	3 minimum

Table J.1. (Continued)

Feeding	None during exposure period	Every 3 days at 1% (v/w) of total organism wet tissue mass period
Overlying water renewal	None during exposure period	Every 3 days during exposure period, immediately before feeding
Control sediment	Uncontaminated sediment of similar physico-chemical properties (grain size, pore-water salinity and pH) to test sediments	Uncontaminated sediment of similar physico-chemical properties (grain size, pore-water salinity and pH) to test sediments
Overlying water	Uncontaminated seawater, adjusted by adding deionised water, to match the salinity of the site where the organism was collected – usually 30 ± 2‰	Uncontaminated seawater, adjusted by adding deionised water, to match the salinity of the site where the organism was collected – usually 30 ± 2‰
Standard endpoints	Metal tissue dose, lipid peroxidation, lysosomal stability, micronuclei occurrence	Metal tissue dose, lipid peroxidation, lysosomal stability, micronuclei occurrence
Additional endpoints	Condition index	Condition index
Test acceptability criteria	>95% survival in controls, maintenance of physico-chemical parameters – dissolved oxygen, salinity, pH, and temperature – within acceptable limits during exposure period	>95% survival in controls, maintenance of physico-chemical parameters – dissolved oxygen, salinity, pH, and temperature – within acceptable limits during exposure period

J.4 Lipid peroxidation biomarker assay

J.4.1 Equipment

- Glass Petri dish.
- Scalpel.
- Polypropylene microcentrifuge tubes 1.5 mL.
- 5 mL glass test tubes and racks.
- Glass beakers.
- Stir plate and magnetic stir bars.
- pH meter.
- Motorised microcentrifuge pellet pestle (Sigma-Aldrich #Z359971/#Z359947).
- Adjustable pipettes.
- Sonicator.
- Centrifuge capable of spinning 1.5 mL microcentrifuge tubes at 10,000 × g at 4°C and glass 5 mL tubes at 1,600 × g at 4°C.
- Spectrophotometer – single cell or plate reader capable of measuring absorbance at 532 nm.
- Water bath at 95°C.

J.4.2 Reagents

A working solution, comprising 1 L of 50 mm potassium phosphate (pH 7), is prepared by mixing 11.2 mL of 1 M potassium dihydrogen phosphate with 38.8 mL of 1 M dibasic

potassium phosphate ($K_2HPO_{4)}$ and making up to volume. The pH is adjusted with 1 M HCl. It is filtered (0.45 μm) and stored at 2–8°C for up to 2 weeks.

All reagents required to perform the TBARS assay are supplied in the assay kits (Zepometrix Corporation #0801192 or equivalent, e.g. Cayman Chemical Co. #10009055). Kits should be stored at 2–8°C until used. All reagents except samples and standards should be at room temperature before beginning the assay. The kit contains sodium dodecyl sulfate (SDS) solution that will take at least 1 h to equilibrate to room temperature and is ready to use once any precipitated SDS is re-dissolved. Briefly heating at 37°C will aid the process and the solution can then be stored at room temperature. Each assay requires 100 μL of SDS solution.

The colour reagent contains thiobarbituric acid powder and acetic acid and sodium hydroxide solutions. To prepare the reagent these three components are diluted and mixed together in sufficient volume for the number of samples plus standards being assayed, as indicated in the protocol supplied with the assay kit. Each assay requires between 2.5 mL and 4 mL of this reagent, depending on the kit. Once mixed, the colour reagent is stable at room temperature for 24 h.

The standard solution contains malondialdehyde-bis (dimethyl acetal) (MDA) in water and is used to prepare the standard curve. The kits provide a set of suggested standard dilutions in a range of 0–100 nmol/mL of MDA equivalents. If necessary the MDA standard can be diluted with water to obtain the starting standard stock solution and this can then be used to prepare a series of 5–8 standards following the standards dilution scheme provided in the kit purchased.

The results are normalised to protein content. Reagents for the protein assay are bovine serum albumin (BSA) (2 mg/mL) solution and Bradford reagent (Coomassie blue dye solution).

J.4.3 TBARS assay

From each chamber, 2–3 depurated organisms are selected at random for lipid peroxidation measurement. The shells are opened, and the tissue is removed and dried by dabbing it on a paper towel, and then it is placed on an inverted glass Petri dish filled with ice. Hepatopancreas tissue is selected (Figs J.3c and J.4b) and excised from the remaining tissue using a scalpel. The hepatopancreas tissue is placed in a 1.5 mL microcentrifuge tube, and chilled buffer is added at 1:5 w/v (i.e. for tissue weighing 0.2 g, 1 mL of buffer is added). The tissue is homogenised in buffer using the pellet pestle for approximately 15–30 s while holding the tube on ice, and then sonicated for 15 s at 40 V over ice and centrifuged at 10,000 × g for 15 min at 4°C. For the TBARS assay, 100 μL of the supernatant is used. If the test cannot be carried out immediately, tissue lysates can be stored at –80°C for up to 1 month. The remaining tissue lysate can be retained for protein analysis.

To 100 μL of sample or standard in a 5 mL glass tube, 100 μL of SDS solution is added and swirled to mix. Then 2.5 mL or 4 mL of colour reagent is added forcefully down the side of each tube. All tubes are placed in a suitable rack and the top of each tube is covered with a glass bead. The samples are incubated in a water bath at 95°C for 60 min, after which they are immediately removed from the water bath and cooled to room temperature in an ice bath for 10 min to stop the reaction. The samples are centrifuged at 1600 × g/3000 rpm for 10–15 min at 15°C. They are transferred to 1 mL cuvettes, or 150 μL, is pipetted in duplicate, from each tube into a 96-well plate. The absorbance is read at 532 nm using a spectrophotometer or plate reader.

The average absorbance of each standard and sample is calculated by subtracting the absorbance value of the blank (standard 0) from itself and all other values for standards

and samples, to produce the corrected absorbance. These corrected absorbance values for each standard are plotted as a function of MDA concentration, and the MDA concentration for each sample is calculated from the slope of the line of the standard curve.

J.4.4 Protein assay

The Bradford protein assay procedure described here is suitable for a microplate spectrophotometer, but can be scaled up for measurement in 1 mL cuvettes using a single beam spectrophotometer. The volume of sample and the Bradford reagent volume, as well as standard concentration ranges, will depend on the concentration of the reagent purchased and the protein concentration of the samples. It is recommended that samples are assayed at a range of dilutions, e.g. 1:10, 1:100, to determine the correct standard curve range to use.

All reagents should be at room temperature. The BSA protein standard is diluted with the same potassium phosphate buffer as is used for the tissue lysate preparation, at concentrations of 0, 50, 100, 250, 500, 750, 1000, 1500 µg BSA/mL.

Using a 96-well plate, 5 µL of each of the diluted BSA standards and samples, and 5 µL of buffer for the blank, is added to duplicate wells. The Bradford reagent (250 µL) is also added to each well. The samples are mixed on a shaker for ≈30 s and then incubated at room temperature for 5–45 min. The absorbance is measured at 595 nm. The protein complex is stable at room temperature for 60 min, so absorbance of all samples must be read before then and within 10 min of each other.

The average absorbance of each standard and sample is calculated by subtracting the absorbance value of the blank (standard 0) from itself and all other values for standards and samples, to produce the corrected absorbance. These corrected absorbance values for each standard are plotted as a function of BSA concentration, and the protein concentration for each sample is calculated from the standard curve.

J.5 Lysosomal membrane stability biomarker assay

J.5.1 Equipment

- Glass Petri dish.
- Scalpel.
- Glass Pasteur pipettes and rubber bulb.
- Flat bottom 24-well plate with cover.
- Small shallow tray to hold ice and 24-well plate.
- Polypropylene microcentrifuge tubes 2 mL.
- 1 mL pipette tips.
- 40 µm nylon mesh.
- Reciprocating shaker, 100–120 rpm.
- 20 µL, 1 mL and 5 mL adjustable pipettes.
- pH meter.
- Air bubbler.
- Aluminium foil.
- Dark incubation chamber at room temperature.
- Centrifuge.
- Compound light microscope with 40× objective.
- Glass slides and coverslips.

J.5.2 Reagents

A calcium- and magnesium-free saline buffer (CMFS) is prepared from 20 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 360 mM NaCl, 12.5 mM KCl and 5 mM tetrasodium EDTA, and adjusted to pH 7.35–7.40 with 6 M NaOH. The solution is filtered through a 0.45 μm screen. A 1.0 mg/mL assay solution of trypsin (powder from bovine pancreas, ≥10,000 BAEE units/mg protein) is prepared by dissolving trypsin in CMFS buffer. The mixture can be frozen (at –20°C) and thawed once, before use.

Fresh stock and working solutions of neutral red dye need to be prepared each day. A 4.0 mg/mL stock solution is prepared by dissolving 4.0 mg of neutral red in 1.0 mL of dimethylsulfoxide in a 2 mL microcentrifuge tube. Immediately before performing the assay, a 0.08 mg/mL working solution is made by mixing 40 μL of the stock solution in 1.96 mL of CMFS buffer in a 2 mL microcentrifuge tube. The solutions should be protected from light by wrapping the tubes in aluminium foil, and they should be kept at room temperature.

J.5.3 Neutral red assay

The CMFS buffer is placed on ice and oxygenated for 15–20 min. The 24-well plate is placed in a shallow tray filled with ice and 500 μL of cold oxygenated CMFS buffer is added to one well per sample number. A depurated bivalve is opened by gently inserting a scalpel blade between the shell valves and cutting the adductor muscle (Fig. J.3b). The soft tissue is removed and placed on an inverted glass Petri dish filled with ice. The hepatopancreas (digestive tract) (Figs J.3c and J.4b) is separated from the remainder of the tissue and washed with 0.5 mL of buffer; a paper towel can be used to remove excess buffer from the Petri dish. Using a scalpel blade, the tissue is finely minced (Fig. J.5), rinsed with CMFS

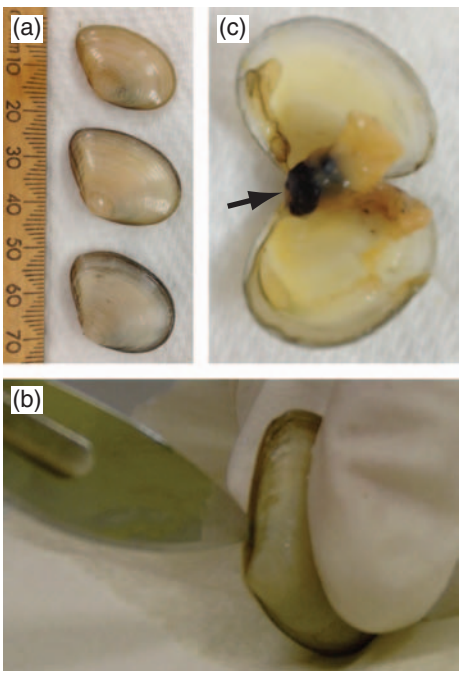

Figure J.3. (a) Depurated *Tellina deltoidalis*; (b) cutting adductor muscle to open the bivalve shell; and (c) soft tissue, with arrow indicating hepatopancreas tissue (photographs: Anne Taylor).

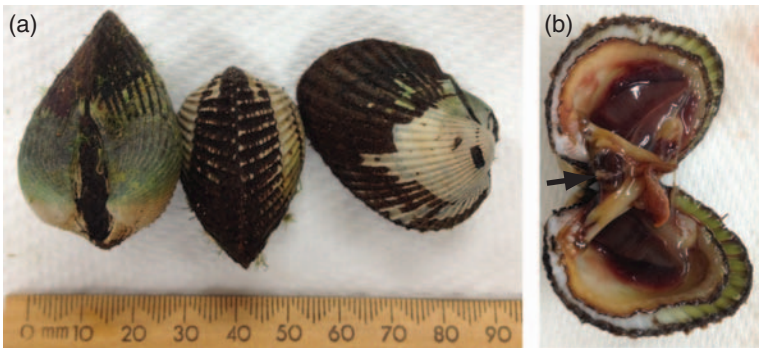

Figure J.4. (a) Depurated *Anadara trapezia*; and (b) soft tissue, with arrow indicating hepatopancreas tissue (photographs: Anne Taylor).

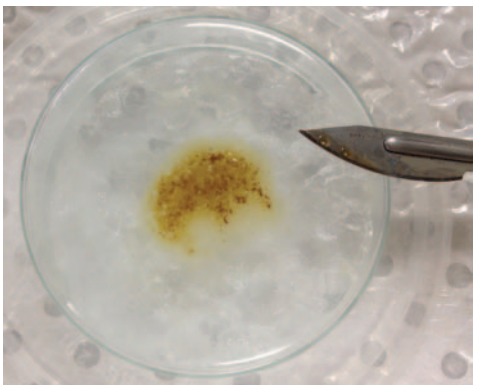

Figure J.5. Minced hepatopancreas tissue on an ice-filled Petri dish (photographs: Anne Taylor).

buffer and then transferred to a well of the 24-well plate containing 500 µL of cold oxygenated buffer (Fig. J.6). When all minced tissue samples are in the well plate, it is covered with the lid and placed on ice, on an orbital shaker, and shaken at 100–120 rpm for 20 min.

To each well, 350 µL of the 1.0 mg/mL trypsin solution is added and the plate is shaken for a further 20 min. A glass Pasteur pipette is used to 'shear' the samples to disaggregate the enzyme digested tissue and release the cells.

The enzyme-digested tissue samples are transferred to the filter apparatus (Fig. J.7) and centrifuged for 5–10 min at 15°C and 200–250 × g. After checking the tips of the centrifuge tubes for cell pellets the tubes are re-centrifuged for a further 5–10 min if necessary. The pipette tip and nylon filter are removed from each centrifuge tube and set aside. The supernatant (liquid) is carefully poured or pipetted from each centrifuge tube, leaving the cell pellets in the tubes, where they are resuspended by adding 1 mL of buffer to each tube and gently mixing. The tubes are centrifuged for 5 min at 15°C and 200–250 × g; the supernatant is removed, and the contents are again washed with buffer and re-centrifuged; the supernatant is removed and the cells are resuspended in 50–100 µL of buffer.

To the above solution, an equal volume of the neutral red 0.08 mg/mL working solution is added and gently mixed through the cells with a plastic pipette tip. The samples are placed in a dark chamber and incubated at room temperature for 1 h. After incubation, a drop of the cell suspension is placed on a glass slide and a coverslip is added. The cells are counted using a light microscope with a 40× objective, counting only large intact cells and

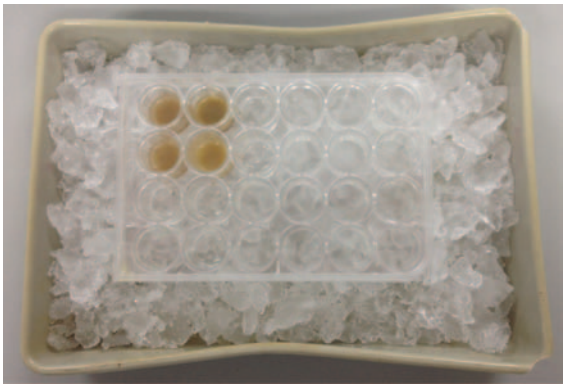

Figure J.6. A 24-well plate on ice with minced tissue in CMFS buffer (photograph: Anne Taylor).

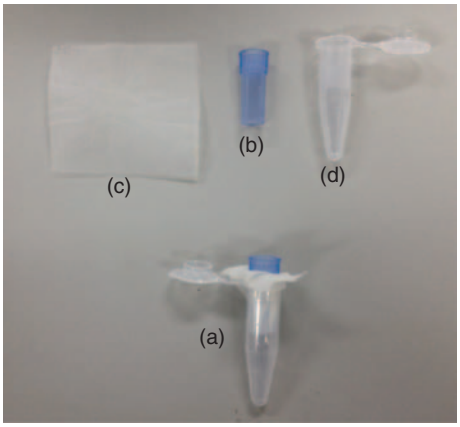

Figure J.7. (a) A 40 µm screen microcentrifuge tube filter apparatus. (b) The apparatus is made by cutting the end off a 1 mL pipette tip. (c) A small square of 40 µm nylon mesh is folded around the cut end of the pipette tip. (d) A 1.5 mL microcentrifuge tube ready to have the assembly pressed into it, to form (a) (photograph: Anne Taylor).

scoring them as 'dye inside the lysosomes' (i.e. stable) or 'dye in the cytosol' (i.e. unstable) (Fig. J.1). After counting 100 cells per sample, the percentage of unstable lysosomes can be calculated. During this process, ~15 min should be allowed between samples for the addition of neutral red, to allow cell counting of the first sample to be completed before the 1-h incubation time of the next sample has elapsed. The time to allow between samples will depend on the speed at which individual operators are able to count cells, and should be adjusted to suit individuals who have more experience of the technique.

J.6 Micronuclei occurrence biomarker assay

J.6.1 Equipment

- pH meter.
- Glass slides and coverslips.
- Fluorescence microscope with appropriate filter for the DAPI – excitation wavelength 350 nm with a 40× objective.

- Glass Petri dish.
- Scalpel.
- Crushed ice.
- Glass Pasteur pipettes.
- 24-well plate with cover.
- Small shallow tray to hold ice and 24-well plate.
- Polypropylene microcentrifuge tubes 1.5 mL.
- 1 mL pipette tips.
- 40 μm nylon mesh.
- Reciprocating shaker, 100–120 rpm.
- 20 μL, 1 mL and 5 mL adjustable pipettes.
- pH meter.
- Air bubbler.
- Centrifuge.

J.6.2 Reagents

- Calcium- and magnesium-free saline buffer (CMFS) – 20 mM HEPES, 360 mM NaCl, 12.5 mm KCl and 5 mM tetrasodium EDTA pH 7.35–7.40.
- Trypsin from bovine pancreas – lyophilised powder, ≥10,000 BAEE units/mg protein.
- DAPI (4,6-diamidino,2-phenylindole dihydrochloride): a 1 mg/mL stock solution is made by mixing 1.0 mg of DAPI in 1.0 mL of deionised water. A working solution is made by adding 10 μL of stock solution to 90 μL of phosphate buffered saline (PBS). The stock solution is kept at 4°C and a fresh working solution is made from it each day.
- Carnoy's Solution is formed by mixing 3 volumes of absolute ethanol with 1 volume of acetic acid; it is stored at 4°C.
- Phosphate buffered saline (PBS): To 800 mL of deionised water are added 8.0 g of sodium chloride, 0.2 g of potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g of potassium dihydrogen phosphate. The pH is adjusted to 7.4 with a 10% solution of HCl and made up to 1 L with deionised water.

J.6.3 Micronuclei assay

Cell suspensions are prepared from hepatopancreas or gill tissues as described above for the lysosomal membrane stability assay. The CMFS-washed cells are resuspended in 1 mL of Carnoy's Solution. Cells are then fixed and can be stored at 2–8°C for 2–4 weeks. A drop of the Carnoy's fixed cell suspension is placed on a glass microscope slide and allowed to air dry. A drop of DAPI working solution is added to the dried cells and covered with a glass coverslip, and the prepared slide is put on the microscope stage. When all lights are turned off, the fluorescence can be observed. The slide is incubated in the dark for 5 min. Then the cells are scored as either normal nuclei (no micronuclei present) or non-normal nuclei (micronuclei present) (Fig. J2), counting 1000 cells per sample and then calculating the number of cells with micronuclei, as a percentage.

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Appendix K: Ecotoxicogenomics: microarray analysis of gene expression in sediment biota

Sharon E. Hook

K.1 Introduction

In many ways, DNA is the blueprint of the cell. It contains all the information that cells need to conduct the diversity of functions of which they are capable. These functions are carried out by proteins. The molecule that is used by cells to make proteins is called mRNA. The regulation of the functional capacity of the cell is complex and includes activating and de-activating proteins and changing transcript degradation rates or translation efficiency, and it is often regulated by mRNA transcript abundance. Many studies have shown that measuring the amounts of mRNA can give you a 'snapshot' of what a cell is doing.

Ecotoxicogenomics involves the use of genomic data to investigate toxicity of environmental contaminants to biota. Gene expression profiling is used as a biomarker and to provide mechanistic information about an organism's exposure to contaminants relative to a control or unexposed organism. Gene expression profiles are determined by looking at the contaminant-induced changes in abundance of a transcript in one condition, relative to a control or unexposed organism.

Microarrays are currently the most common tools for evaluating changes in gene expression, although RNA-sequencing (RNA-Seq) studies based on next generation sequencing are rapidly gaining in popularity (Mehinto *et al.*, 2012). A typical microarray comprises an ordered array of DNA spots on a glass slide, each containing picomoles of a specific DNA sequence, known as a probe. The probe may be a short oligonucleotide, a long oligonucleotide, or a full cDNA (complementary DNA) sequence. To perform microarray-based gene expression analysis (Fig. K.1), the RNA is extracted from the specific organism being investigated. The RNA is reverse-transcribed into cDNA and sometimes amplified using a polymerase chain reaction (PCR). The cDNA is labelled with different fluorescent dyes, then hybridised onto the array. The spots on the array each represent a probe for a specific gene.

In microarray studies, RNA from control and test samples is labelled with different fluorescent dyes (e.g. green for control and red for treatment) and hybridised onto the array. In the example illustrated in Fig. K.2, each spot has a short probe which is specific for a single gene. Spots that appear dark are genes that are not 'expressed' or activated. Spots that

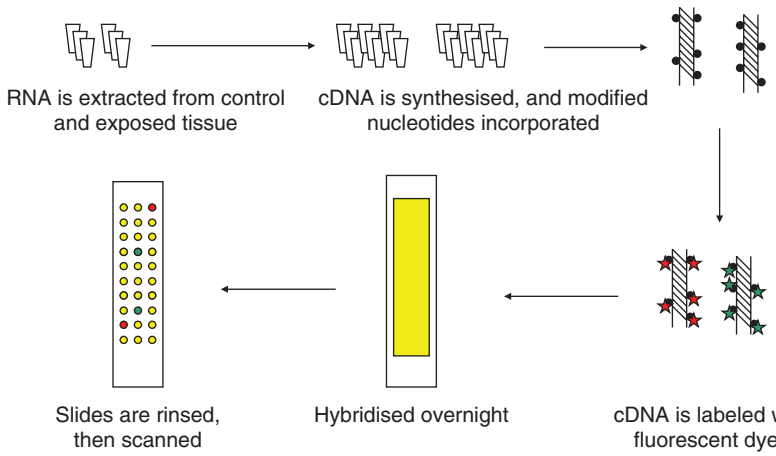

Figure K.1. Schematic diagram of the steps involved in microarray-based genomic analysis.

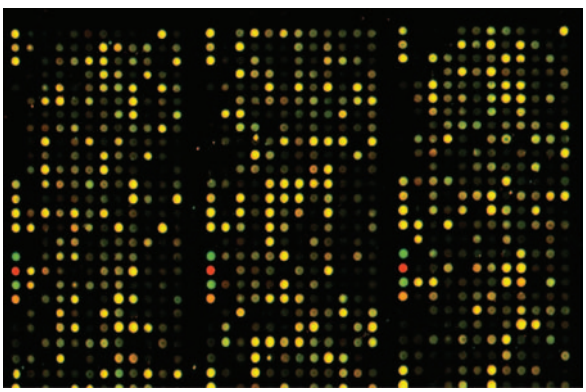

Figure K.2. A close-up of a section of a microarray slide.

appear yellow are expressed roughly equally in control and exposed treatments. Spots that appear red are genes that are expressed more in the exposed treatments, and green spots are less abundant in exposed treatments.

In microarray data analysis, the fluorescence in the green and red wavelengths for each spot is measured, to determine whether the abundance of a given transcript is greater in the treatment relative to the control. The ratio of fluorescence for each probe provides a semi-quantitative measure of the relative abundances of the specific transcript in each treatment.

The concentration at which exposure to a contaminant results in no significant changes in gene expression has been defined as the 'no observed transcriptional effect level' (NOTEL) (Lobenhofer *et al*., 2004). This concentration is often far below the concentrations at which whole organism toxic effects are seen (Poynton *et al*., 2007; Poynton and Vulpe, 2009).

This appendix gives a brief description of the procedure involved in preparing a sample for microarray analysis. It does not describe the experimental design phase, i.e. choosing the most appropriate exposures, doses, and sampling times. It also does not provide the exact protocols (all of which are available online and come with the reagents to be purchased), and hence it does not cover the health and safety risks. Note that some commonly used reagents

are quite caustic and toxic, and that the Material Safety Data Sheet should be carefully reviewed before any work is planned.

It is highly recommended that microarray hybridisations be performed at a specialist laboratory or service provider, both because the acquisition of the necessary equipment is expensive and equipment quickly becomes obsolete, and because the procedures are quite detailed and vary from array type to array type. Finally, as detailed below, working with RNA is challenging; it is recommended that time be spent in an established molecular laboratory before work is undertaken.

The same basic principles apply to an RNA-Seq experiment (in which cDNA is sequenced directly through using high output next generation sequencers), but as this is emerging technology the requirements and procedures change rapidly. Contacting the proposed sequencing centre is recommended to determine current specifications before planning work on RNA-Seq experiments.

K.2 Method

K.2.1 Test organisms

When selecting a test species, in addition to all the other factors that normally go into species selection, it is necessary to determine whether the selected species has the necessary genomic underpinning to allow for the creation of a microarray. Does the organism to be studied have enough sequences available to allow for microarray construction, or should an RNA-Seq experiment be performed instead? Another important consideration is whether sufficient quantities of tissue can be obtained from the selected species to allow for extraction of 100 ng of RNA per replicate, or if pooling will be required. To avoid confounding the data interpretation, the control organisms and the exposed organisms should be identical in every variable except contaminant exposure.

K.2.2 Equipment

The following equipment may be required.

- Chemical fume hood.
- Bead beater, fast preparation system, or similar tissue homogeniser.
- Mortar and pestle and liquid nitrogen Dewar flask.
- Refrigerated centrifuge capable of holding 2 mL tubes and spinning at up to 12, 000 × g.
- Dedicated RNA workspace with dedicated pipettes.
- A 37°C incubator.
- Spectrophotometer.
- Acrylamide gel rig or a micro-electrophoresis device.

The exact equipment required will vary depending on the RNA extraction protocols.

K.2.3 Method

Preparing the workspace

RNA is highly unstable and can completely degrade in seconds. Contamination from ubiquitous sources of RNases (ribonucleases – types of nucleases that catalyse the degradation of RNA) in the environment (including human skin cells, bacteria and fungi) is a major issue, so training in a laboratory with experience with RNA, ideally of the selected organism(s), is

recommended. There are some helpful hints online, but these are not a substitute for working with someone having experience, ideally with sediments.

Work with RNA must be performed in a dedicated workspace that is kept free of dust and material that may contain bacteria and fungal spores. This workspace must be cleaned before work is started (ideally with 70% ethanol and RNaseZap® or similar reagent). UV sterilisation is also recommended. All procedures must be performed wearing gloves to protect samples from the RNases normally present in skin. All procedures must be carried out using aerosol-resistant micropipette tips. All consumables must either be RNase-free or RNase-cleaned. There should be a set of pipettes that is dedicated for RNA work.

RNA extractions

Ideally, the organism to be studied will have depurated the sediments from its gut contents before it is harvested. At the very least, excess sediment should be rinsed from the tissue before the organism is sacrificed. The organism(s) to be used must be instantly killed and the tissues excised and either immersed in liquid nitrogen or preserved using a preservative designed for RNA (such as RNAlater® or RNAprotect®). The entire procedure should take less than 10 min to ensure that RNA is not hydrolysed (a normal part of necrosis). Tissues that are flash frozen must stay at or below –80°C until samples are processed.

The RNA extraction protocol should be optimised before starting experimental work. Lysing the tissue without hydrolysing the RNA is the critical step. Some options include use of a fast preparation system or bead beater (for most animal tissues) (e.g. Hook *et al*., 2006, 2014a), or involve grinding tissue with a mortar and pestle that has been super-cooled with liquid nitrogen (for bivalve tissue) (e.g. Hook *et al*., 2014b), and a combination of a fast preparation or bead beating system and a lysis buffer (described in Hook and Osborn, 2012; Osborn and Hook, 2013) (for plant tissue). Optimising extraction protocols is an iterative process and approaches that work well for another organism may not work well for the selected species.

After tissue lysis, the material is homogenised. This is performed using a phenol:chloroform extraction protocol, using TRIzol® or similar reagents, or using an RNA extraction kit (such as a QIAGEN RNeasy kit, or a MO BIO Power RNA kit). A popular approach is to combine solvent–based extraction approaches with a column-based clean up procedure (such as Invitrogen's TRIzol® Plus RNA Purification Kit). Just like tissue lysis, effective RNA extractions need to be optimised for the organism you have chosen.

Once RNA is extracted, genomic DNA contamination needs to be removed. This can be done using a commercially available DNAse (such as Ambion® TURBO™ DNA-*free*™).

Sediments are typically high in humic acids and other materials that may inhibit downstream applications. To remove this material, the DNA must be precipitated (either via ethanol precipitation or vacuum concentration) and rinsed with ethanol to remove salts and other impurities.

Designing and selecting an array

For many common biomedical test species (e.g. human, rat, mouse, fruit fly, zebrafish), commercial arrays are available; see Agilent (www.agilent.com.au) or Affymetrix (http://www.affymetrix.com) for more details. For other species, if sequence information is available via the US National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) or another database, a custom microarray can be designed (see the Agilent 'e array' website). If no sequence information is available, changes in gene expression may be best measured using an approach based on RNA-Seq (see Mehinto *et al*., 2012).

It is highly recommended that a commercial service provider be used for microarray hybridisations for reasons outlined above. Examples include the Ramaciotti Centre for Genomics at the University of New South Wales, in Sydney, Australia, and the Australian Genome Research Facility Ltd, which has offices in all states and territories of Australia.

Microarrays can be purchased containing probes for specific taxa, e.g. zebrafish, or they can be custom designed and built using transcripts obtained from next-generation sequencing experiments or sequences downloaded from on-line repositories, such as the Gene Expression Omnibus at the NCBI (http://www.ncbi.nlm.nih.gov/geo/).

K.2.4 Quality assurance

For RNA to be considered of sufficient quality to be used, it must meet the following criteria:

1. There must be sufficient RNA for analysis. Most array protocols require at least 25 ng/μL of total RNA, and many require 100 ng/μL.
2. The RNA must be free from protein contamination. This is typically measured using the 260/280 ratio (nucleic acid/protein), determined spectrophotometrically, with a minimum ratio of 2.0. A microfluidic device such as a nanodrop™ is often used to measure RNA concentration.
3. The RNA must not be degraded. To determine the degree of degradation, aliquots of RNA are visualised either using a bioanalyzer, a multiNA, or a denaturing acrylamide gel. The quantity and sharpness of 28S and 18S rRNA sub-units (these should be roughly equal) is used to determine RNA quality.

K.2.5 Data analysis

The analysis of microarrays is discussed by Draghici (2003). Some factors discussed by Draghici, but not here, include (but are not limited to) background correction, normalisation of different dye intensities, inter- and intra-array variability, match/mismatch calls, criteria for differential expression and multiple test correction. Most analyses of microarray data are either conducted using commercially available software (such as Agilent's GeneSpring®) or via BioConductor (http://www.bioconductor.org) in R.

The following guidelines are for two-colour Agilent-type microarrays. The procedure to generate a gene list, or a list of transcripts with abundances that significantly differ from control, using GeneSpring® or similar software, is described below.

LOWESS normalisation

A LOWESS normalisation is performed to correct for differences in dye intensity, dye incorporation and batch effects. The algorithm is described in more detail in Draghici (2003), but the underlying assumption is that the majority of transcripts on the array will not be altered in response to treatment. If a targeted array (with only transcripts that are expected to respond to the treatment) or very high doses (where the organism is dying and shutting down transcription in general) are used, this assumption is inappropriate and the normalisation step should not be used (Draghici, 2003).

Fluorescence must be greater than 2 standard deviations from background

Since two-colour arrays are presented as ratios, very low numbers in the denominators are essentially dividing by zero, and will produce spurious results and very high variability. Consequently, two-colour readings are considered 'signal' and not 'noise' if their fluorescence values are not greater than 2 standard deviations from mean background fluorescence.

Mean background fluorescence is typically subtracted from the fluorescence in each channel (Draghici, 2003).

Fold change or other means of reducing the number of comparisons

A multiple test correction is typically performed (Draghici, 2003) because of the dimensionality of the data (e.g. high number of features on an array and the low number of replicates performed in comparison (a 10,000 feature array often has 5–10 replicates)). A *P* value of 0.05 for a 20,000 feature array would lead to 1000 false positives. However, studies using *P* values based on multiple test correction, as the only means of selecting differentially expressed genes, have low replicability for which genes are considered 'significant' (see Shi *et al*., 2008; Hook *et al*., 2010). Instead, a filter (for example, the absolute value of fold change must be higher than 2 in 4 out of 5 replicates) is typically applied to gene lists before significance testing. This decreases the number of comparisons to be made, and increases the reliability of significant lists (Williams *et al*., 2008).

t-test with multiple test correction

Once gene lists have been filtered, a *t*-test or analysis of variance with an appropriate multiple test correction is applied. For guidance in selecting the tests and correction, see Draghici (2003).

At the end of analysis, it is recommended that the number of differentially expressed features obtained be compared to the number that would be expected due to chance alone, to determine if the false positive rate is within the acceptability criteria. An example of the outcomes of these comparisons can be found in Osborn and Hook (2013).

Gene lists may contain hundreds of genes and no publication can reasonably contain a table with 100+ entries. To visualise the data, transcript abundance levels are usually graphically presented using heat maps. In Fig. K3, each column represents an experimental

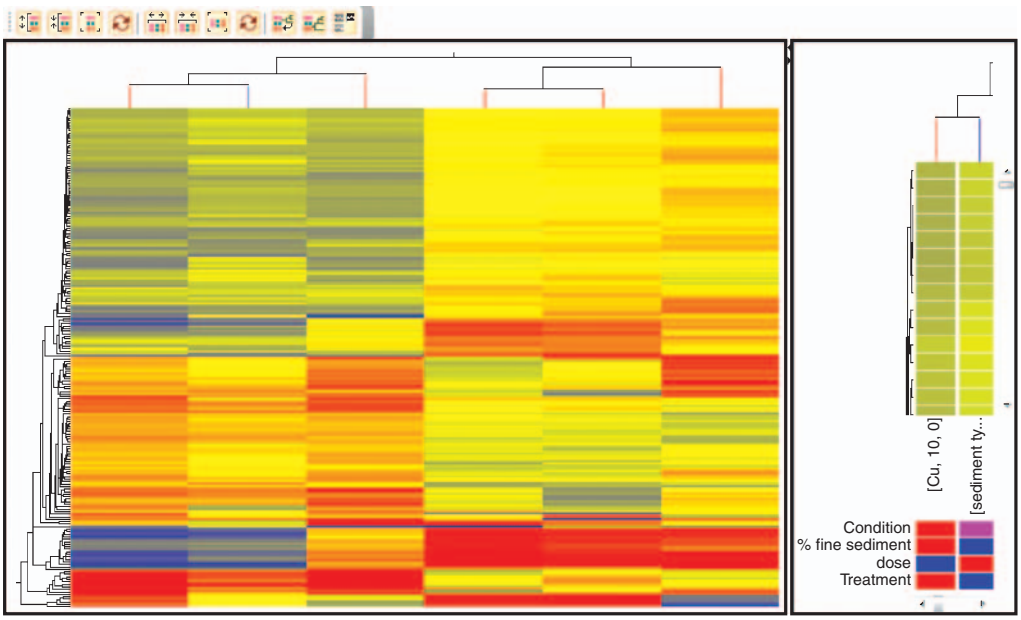

Figure K.3. A heat map summarising the microarray data produced from a transcriptomic study.

treatment and its replicates, and each horizontal row represents a spot on the array. The degree to which each entry in the resulting matrix is red or blue represents the ratio of red/green dye, with red indicating that gene is being more expressed in the treatment when compared to the control. By using a hierarchical clustering algorithm to group the genes and treatments, the genes that are expressed similarly, and that are likely to have a similar function in response to the contaminant, can be determined. This functional information can then be used to produce transcriptomic profiles of how the test organism responds to particular stressors under specific conditions.

K.2.6 Data storage and submission to public databases

Microarray data are typically gigabytes of information and will need to be archived on a permanent drive so that subsequent analysis may be performed if necessary. It is also typically a requirement for publication that MIAME (minimum information about microarray experiments; Brazma *et al*., 2001) compliant data be submitted to a public database, such as the NCBI Gene Expression Omnibus (see above). Instructions can be found at <www.ncbi.nih.gov/geo>.

Important note: Mention of a specific trade name or commercial service in this appendix does not imply endorsement. These entities are mentioned for purposes of illustration only.

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Glossary of terms and acronyms

AAS: Atomic absorption spectrometry
ACR: Acute to chronic ratio (for toxicity)
Acute toxicity: An adverse lethal (mortality) or sub-lethal (e.g. bioluminescence inhibition) effect from a toxicant that occurs as a result of an exposure period that is short relative to the organism's life span
AEM: Dilute-acid extractable metal (1 M HCl). This is equivalent to SEM.
AFS: Atomic fluorescence spectrometry
Algae: Aquatic plants that do not have root structures or flowers. Microalgae (also called phytoplankton) are microscopic. Macroalgae can be seen without magnification
Amphipod: A malacostracan crustacean of the order Amphipoda; typically these live in or on sediment
ANOSIM: Analysis of similarities
ANOVA: Analysis of variance
ANZECC: Australian and New Zealand Environment and Conservation Council
Aquatic ecosystem: Any water environment, from an ephemeral pond to the ocean, in which plants and animals interact with the chemical and physical features of the environment
ARMCANZ: Agriculture and Resource Management Council of Australia and New Zealand
ASE: Accelerated solvent extraction (for extraction of organic contaminants from samples)
AVS: Acid volatile sulfide(s); the acid-soluble sulfide concentration in an aquatic sediment
BEDS: Biological effects database for sediment
Benthic organisms: Referring to biota living in or on the sediments of aquatic habitats
Bioaccumulation: A general term describing a process by which chemical substances are accumulated by aquatic organisms from water directly or through consumption of food containing the chemicals
Bioassay: A test used to evaluate the relative potency of a chemical by measuring its effect on a living organism relative to a control or reference
Bioavailable: Able to be taken up by organisms
Biodiversity: The variety and variability of living organisms and the ecological complexes in which they occur
Biomagnification: The result of the processes of bioaccumulation by which tissue concentrations of bioaccumulated chemicals increase as the chemical passes up through two or more trophic levels. Compounds that are biomagnified have higher tissue concentrations in predators than they do in prey. The term implies an efficient transfer of chemicals from food to consumer so that the residue concentrations increase systematically from one trophic level to the next
Biometrician: A person having skills in biometrics (biological statistician)
Biomimetic: An approach, method or device that mimics a biological process (e.g. a chemical extraction that aims to mimic the extraction of a contaminant, such as by gut fluids when the contaminant is ingested)

Bivalve: A mollusc with a shell in two parts, hinged together. Clams, oysters and mussels are examples of bivalves
BTEX: Benzene, toluene, ethylbenzene, xylene
Chelex: A resin with strong metal-binding properties commonly used in chemical analyses
Chronic toxicity: Adverse effects over a significant portion of the organism's life span; e.g. effects on growth and reproduction
Clastogenic: An agent causing breaks in chromosomes
Clean-room environment: A dust-free filtered-air environment, typically created using HEPA filters
COC: Contaminant of concern
Coelomic fluid: Fluid that exists within the body cavity that surrounds the digestive tract and other organs
Community: Assemblage of organisms characterised by a distinctive combination of species occupying a common environment and interacting with one another
Community composition: All the types of taxa present in a community
Concentration: The quantifiable amount of a substance in water, food or sediment, per unit volume or weight
Contaminants: Biological or chemical substances or entities, not normally present in a system, capable of producing an adverse effect in a biological system, seriously injuring structure or function
Contaminated sediment: A sediment containing chemical substances at concentrations above background concentrations and above the ANZECC/ARMCANZ guideline values
Control: Part of an experimental procedure that is ideally exactly like the treated part except that it is not subject to the test conditions. It is used as a standard of comparison, to check that the outcome of the experiment is a reflection of the test conditions and not of some unknown general factor
Control sediment: A sediment that is sufficiently free of contaminants that it will not cause effects to test organisms. Generally, a control sediment will have physico-chemical parameters similar to those of the test sediments
COPC: Contaminant of potential concern
Copepod: A small crustacean found in the sea and nearly every freshwater habitat, which can be either planktonic (drifting in sea waters), or benthic (living on the sediments)
Coprophagous: Organisms that consume faeces – their own or those of other organisms
CRM: Certified reference materials (also called standard reference materials) used as part of the analysis QA/QC
Cytosolic: Referring to the water-soluble components of cell cytoplasm, constituting the fluid portion that remains after removal of the organelles and other intracellular structures
Dalton: A standard unit for indicating mass on the atomic or molecular scale (a kilodalton is equal to 1000 atomic mass units)
DDD: Dichlorodiphenyldichloroethane
DDE: Dichlorodiphenyldichloroethylene
DDT: Dichlorodiphenyltrichloroethane
DET: Diffusive equilibrium in thin films (an equilibrium technique for passive sampling of dissolved metals)
Detection limit (DL): The lowest concentration of a substance which when processed through the complete analytical method produces a signal that has a 99% probability of being different from the blank, calculated as three times the standard deviation of the blank.
DGT: Diffusive gradients in thin films (a kinetic technique for passive sampling of dissolved metals)

Dipteran larvae: Larvae of insects of the order Diptera (includes mayflies, dragonflies, damselflies and caddisflies)
DO: Dissolved oxygen
DOC: Dissolved organic carbon
Dry shipper: A custom-built aluminum cryogenic Dewar flask that uses liquid nitrogen and is validated to maintain a stable temperature below –150°C when samples are transported over long durations (for example, shipment lasting 5 days or more)
DTA: Direct toxicity assessment
Ecogenomics: The examination of genetic (DNA) materials in environmental samples for the purpose of identifying the organisms present
Ecotoxicology: The science dealing with the adverse effects of chemicals, physical agents and natural products on populations and communities of living organisms
EC10, EC20, and EC50: The toxicant concentration(s) that are expected to cause one or more specified effects in 10%, 20% and 50%, respectively, of a group of organisms under specified conditions
E_h: Redox potential
Epifauna: Animals living on the surface of the seabed or a riverbedor attached to submerged objects or aquatic animals or plants
EqP: Equilibrium partitioning
ERL: Effects range low
ERM: Effects range median
ESB: Equilibrium partitioning sediment benchmark
Eukaryote: An organism consisting of a cell or cells in which the genetic material is DNA in the form of chromosomes contained within a distinct nucleus. Eukaryotes include all living organisms other than the eubacteria and archaea
FACR: Final acute to chronic ratio
FAV: Final acute value
FCV: Final chronic value
F_{OC}: Fraction of organic carbon
f-SSD: Field-based species sensitivity distribution
f-CSD: Field-based community sensitivity distribution
GC/MS: Gas chromatography/mass spectrometry
GPS: Global positioning system
Guideline: Numerical concentration limit or narrative statement to support and maintain a designated water use
HC: Hazardous concentration, usually relating to a given percentage of species; e.g. HC5 is the concentration hazardous to 5% of species
HDPE: High-density polyethylene (a high quality plastic; suitable for storage of samples analysed for metals or inorganic contaminants)
HG-AFS: Hydride-generation atomic fluorescence spectrometry
HOC: Hydrophobic organic contaminant
HPLC: High-performance liquid chromatography
IC50: A toxicant concentration that would cause a 50% reduction in a non-quantal measurement such as fecundity or growth
ICPAES: Inductively coupled plasma atomic emission spectrometry
ICPMS: Inductively coupled plasma mass spectrometry
Index (indices): Composite value(s) that can give a quick ranking to a waterbody or other ecosystem feature, derived via a formula that combines measurements of important ecosystem characteristics; typically used to rank 'health' or naturalness

Indicator: Measurement parameter or combination of parameters that can be used to assess the quality of water
Infauna: Aquatic animals, such as clams or burrowing worms, which live in the sediment
Invertebrates: Animals lacking a dorsal column of vertebrae or a notochord
Iteroparous: Having multiple reproductive cycles in a lifetime
K_D: Sediment–water partition coefficient
K_{OC}: Organic carbon based sediment–water partition coefficient
K_{OW}: Octanol–water partition coefficient
Laminar flow cabinet: A cabinet used to create a dust-free filtered-air environment, to minimise contamination when preparing equipment for trace analyses and handling trace analysis samples
LC50: A toxicant concentration that is expected to be lethal to 50% of a group of organisms under specified conditions
LDPE: Low-density polyethylene
LT50: The time taken for a single toxicant concentration to be lethal to 50% of a group of organisms under specified conditions
Level of protection: The acceptable level of change from a defined reference condition
Loci: Locations along a sequence of DNA or RNA. Positions along a sequence where a particular gene of interest lies
LOE: Line of evidence
LOI: Loss on ignition
LOD: Limit of determination (analogous to LOR and PQL)
LOR: Limit of reporting (analogous to LOD and PQL)
LOEC (Lowest observed effect concentration): The lowest tested concentration of a material (toxicant) at which organisms are adversely affected compared to control organisms
MDS: Multidimensional scaling
Measurement parameter: Any parameter or variable that is measured to find something out about an ecosystem
Mensurative experiment: An experiment in which the experimenter does not manipulate the system of study, but instead collects data about the system in its natural state with all the inherent variables
Metabarcoding: The DNA-based identification of multiple species from a single complex environmental sample. It uses universal PCR (polymerase chain reaction) primers to mass-amplify DNA barcodes from collections of organisms. Each sample is allocated a unique barcode and sequenced using high-throughput sequencing
Mitotic spindle: A spindle-shaped structure that develops outside the nucleus of a cell during mitosis (a part of the cell cycle process by which chromosomes in a cell nucleus are separated into two identical sets of chromosomes, each in its own nucleus)
Multivariate: Relating to more than one variable, especially in observations and statistical analyses. Inclusion of biological variables and environmental variables simultaneously to describe patterns and relationships of the system
nMDS: Non-metric multidimensional scaling
NOAA: United States National Oceanic and Atmospheric Administration
NOEC (No observed effect concentration): The highest tested concentration of a material (toxicant) at which organisms are unaffected, as compared to control organisms
Nondetect (ND) values: Censored data values in relation to computed decision statistics (e.g. 95%UCL), often resulting from measurements being below the detection limit for the analytical method. Within environmental datasets there may potentially be many nondetects with different limits for the same parameter

Non-tubicolous: An organism that does not live in a self-constructed tube (as used by a marine worm)
NWQMS: National Water Quality Management Strategy
OC: Organochlorine
Organism: Any living animal or plant; anything capable of carrying on life processes
Outliers: Measurements (usually larger or smaller than the majority of the data values in a sample) that are not representative of the population from which they were drawn. The presence of outliers distorts most statistics if used in any calculations
Overlying water: The water above the sediment at a collection site or in a test chamber
Oxidation: The combination of oxygen with a substance, or the removal of hydrogen from it; or, more generally, any reaction in which an atom loses electrons
PAH: Polycyclic aromatic hydrocarbon
PCB: Polychlorinated biphenyl
PE: High probability of effects
PED: Polyethylene device (for passive sampling of organic contaminants)
Peeper: A passive sampler for collecting pore water
PEL: Probable effects level
PERMANOVA: Permutational analysis of variance
PES: polyether-sulfone (a material used for filter membranes of peepers)
Pesticide: Substance or mixture of substances used to kill unwanted species of plants or animals
pH: The intensity of the acidic or basic character of a solution, defined as the negative logarithm of the hydrogen ion concentration of a solution
Phase: Distinct state of matter (solid, liquid or gas) which in aquatic systems comprises sediment, water and air
Phytoplankton: Plants or photosynthetic bacteria, usually microscopic, floating in aquatic systems. Also called microalgae
Polychaete worm: Chiefly marine annelids that inhabit benthic environments; they possess both sexes and have paired appendages (parapodia) bearing bristles
POM: Polyoxymethylene
Pore water: The water that occupies the space between and surrounds individual sediment particles in an aquatic sediment (often called interstitial water)
PQL: Practical quantification limit, analogous to limits of determination (LOD) and level of reporting (LOR)
Principal component analysis (PCA): A method for capturing gradients that encompass multiple variables
Principal coordinate analysis (PCO, Multidimensional scaling, MDS): A method to explore and to visualise similarities or dissimilarities of data. It starts with a similarity matrix or dissimilarity matrix and assigns for each item a location in a low-dimensional space
PTFE: Polytetrafluoroethylene (a high quality plastic for storage of samples analysed for metals or inorganic contaminants)
Proteomics: The study of the total protein content of a cell
Pulmonate snail: A broad category of aquatic snails that breathe via a lung-like pulmonary cavity
QA/QC: Quality assurance/quality control
Quality assurance (QA): The implementation of checks on the success of quality control (e.g. replicate samples, analysis of samples of known concentration)
Quality control (QC): The implementation of procedures to maximise the integrity of monitoring data (e.g. cleaning procedures, contamination avoidance, sample preservation methods)

Recolonisation: A manipulative field experiment to test location-specific relationships, studying the organisms that colonise sediments that have previously been defaunated

Redox: Simultaneous (chemical) reduction and oxidation; reduction is the transfer of electrons to an atom or molecule, whereas oxidation is the removal of electrons from an atom or molecule

Redox potential: A measure of the oxidation–reduction potential (ORP) of sediments. The redox potential is often reported as E_h (versus the normal hydrogen electrode)

Reference condition: An environmental quality or condition that is defined from as many similar systems as possible (including historical data) and used as a benchmark for determining the environmental quality or condition to be achieved and/or maintained in a particular system of equivalent type

Reference sediment: A sediment, generally collected near the study site, which is used to assess the effect of sediment and overlying water conditions in the absence of the material(s) (contaminants, toxicants) of interest

Reference toxicant test: A test conducted with a reference chemical (toxicant) to assess the sensitivity of the test organisms

Risk: A statistical concept defined as the expected frequency or probability of undesirable effects resulting from a specified exposure to known or potential environmental concentrations of a material, organism or condition. A material is considered safe if the risks associated with its exposure are judged to be acceptable. Estimates of risk may be expressed in absolute or relative terms. Absolute risk is the excess risk due to exposure. Relative risk is the ratio of the risk in the exposed population to the risk in the unexposed population

Salinity: The presence of soluble salts in water or soils

Sediment: Unconsolidated mineral and organic particulate material that has settled to the bottom of aquatic environments

SEM: Simultaneously extracted metals

Solution concentration: Concentration of chemicals in the liquid phase

Spatio-temporal: Consideration of both space and time

Speciation: Measurement of different chemical forms or species of an element in a solution or solid

Species: Generally regarded as a group of organisms that resemble each other to a greater degree than members of other groups and that form a reproductively isolated group that will not normally breed with members of another group. (Chemical species are differing compounds of an element.)

Species richness: The number of species present (generally applied to a sample or community)

Spiked sediment: A sediment to which a material has been added for experimental purposes

SPMD: Semipermeable membrane device (for passive sampling of organic contaminants)

SPME: Solid phase microextraction fibre (for passive sampling of organic contaminants)

SQGV: Sediment quality guideline value for a specific contaminant, which when exceeded may lead to additional studies to confirm or deny the possibility of biological impacts of that contaminant

SQGV-high: Upper sediment quality guideline value, equivalent to the ERM

Statistical power: The ability of a statistical test to detect an effect given that the effect actually exists

Stressors: The physical, chemical or biological factors that can cause an adverse effect on an aquatic ecosystem as measured by the condition indicators

SSD: Species sensitivity distribution

STU: Sediment toxic unit

Sub-lethal: A toxic or deleterious effect below the level of exposure that causes death

Taxon (taxa): Any group(s) of organisms considered sufficiently distinct from other such groups to be treated as a separate unit or units (e.g. species, genera, families)
Taxa richness: Number of taxa present
TBT: Tributyltin
TC: Total concentration
TE: Threshold for effects
TEL: Threshold effects level
TEC: Threshold effects concentration
TIE: Toxicity identification evaluation
TITAN: Threshold Indicator Taxa Analysis. The analysis examines how each taxon responds to an environment gradient
TOC: Total organic carbon
Toxicant: A chemical capable of producing an adverse response (effect) in a biological system, seriously injuring structure or function or producing death. Examples include pesticides, heavy metals and biotoxins
Toxicity: The inherent potential or capacity of a material to cause adverse effects in a living organism
Toxicity test: The means by which the toxicity of a chemical or other test material is determined. A toxicity test is used to measure the degree of response produced by exposure to a specific level of the test material (or concentration of chemical)
TPH: Total petroleum hydrocarbon
TPM: Total particulate metal (digestion with strong acids)
Transcription: Making an mRNA copy of DNA, most often instructing a cell to make a particular protein product. Also called 'gene expression'
Transcripts: Copies of mRNA instructing a cell to make a particular protein product
Transcriptomics: The study of all the expressed genes or all mRNA in a cell type. Also called 'Global gene expression'
Transitional water: Region of waterway where salinity changes considerably, daily or seasonally (the freshwater end of an estuary)
Triploid oysters: Oysters having three sets of chromosomes not two. Triploid oysters are functionally sterile and do not spawn
Trophic level: A notional stage in the 'food chain' that transfers matter and energy through a community; primary producers, herbivores, carnivores and decomposers each occupy a different trophic level
Trophic transfer: Transfer of accumulated contaminants from one level of the food chain to the next higher level
TV: Trigger value (previous term for SQGV)
UCL: Upper confidence limit (usually a 95% UCL is calculated as the 95% upper confidence limit of the mean)
Univariate: Relating to a single variable, especially in statistical analysis where each variable is explored separately. Quantification of one variable
Uptake: A process by which materials are absorbed and incorporated into a living organism
Whole sediment: The sediment and associated pore water that have had minimal disturbance or manipulation
WQG: Water quality guideline
WOE: Weight-of-evidence

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