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Integrated marine environmental monitoring of chemicals and their effects

Editors

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1 Introduction to guidance on integrated monitoring and assessment of chemicals and biological effects

Ian M. Davies and Dick Vethaak

The marine environment is the ultimate repository for complex mixtures of persistent chemicals. Consequently, organisms are exposed to a range of substances, many of which can cause metabolic disorders, an increase in disease prevalence, and, potentially, effects on populations through changes in, for example, growth, reproduction, and survival. Through much of the history of marine pollution research and monitoring, chemical and biological field studies have often remained largely independent of each other. There are many publications describing the distribution of hazardous substances in the marine environment and, equally, many describing the perturbations of species or communities as a consequence of exposure to hazardous substances. However, it is now generally agreed that the assessment of environmental quality, and the design and monitoring of measures to improve environmental quality, are best undertaken on the basis of combinations of appropriate sets of chemical and biological measurements. There have been many apparent barriers to following this strategy; for example, there has been a lack of coherent frameworks to guide the selection of organisms, substances, and biological effects measurements for monitoring and assessment programmes; and a lack of guidance on methodology, particularly guidance on the interpretation of data in terms of biological (environmental) significance and on how suites of chemical and biological measurements can be integrated to give the added power and scope of assessment promised by such a multifactorial approach.

The pressure to clarify an integrated approach to biological effects and chemical monitoring increased following the OSPAR Quality Status Report (QSR) 2010 process, and the requirements of Descriptor 8 under the Marine Strategy Framework Directive (MSFD). OSPAR, together with HELCOM, have agreed on an ecosystem approach to managing the marine environment, under which OSPAR has committed itself to monitoring the ecosystems of the marine environment in order to understand and assess the interactions between, and impact of, human activities on marine organisms. Integrated monitoring and assessment of contaminants in the marine environment and their effects will contribute effectively to the integrated assessment of the full range of human impacts on the quality status of the marine environment, as part of the ecosystem approach. MSFD Descriptor 8 of "Good Environmental Status" (contaminant concentrations do not give rise to biological effects) very clearly points towards integrated chemical and biological assessment methods.

The fundamental issues were crystallized in a request (2008/8) from the OSPAR Commission to the International Council for the Exploration of the Sea (ICES) in 2008. It included completing the development of OSPAR Joint Assessment and Monitoring Programme (JAMP) guidance for integrated monitoring of chemicals and their biological effects through preparing technical annexes on groups of biological effects methods to be deployed to address specific questions. This should provide guidance on recommended packages of chemical and biological effects methods for monitoring on a determinant basis to ensure that chemical and biological methods were well matched, that chemical analysis underpinned biological effects monitoring, and that monitoring and assessment are both carried out in an integrated way.

An integrated approach to monitoring is based on the simultaneous measurement of contaminant concentrations (in biota, sediments, and, in some cases, water or passive samplers), biological effects parameters, and a range of physical and other chemical measurements to permit normalization and appropriate assessment. Integrated monitoring of contaminants and their effects requires coordination of field sampling and sample-handling techniques, utilizing the same species/population/individual for both types of measurement, from the same area and sampling within the same time-frame. Furthermore, a set of supporting parameters should be measured at the same time, and such data have to be available for use in the final assessment, because biological effects may be influenced by, for example, temperature, stage of maturation, or size. Integration of effort in this way will yield additional information in a cost-effective manner, while also reducing the interannual variance of the data. The integration of data assessment across a range of chemical and biological measurements requires a coherent suite of assessment criteria that address the aims and objectives of both the monitoring programme and the underlying drivers for improved environmental quality and sustainable use of the sea.

This report presents the advice given to OSPAR by ICES through the work of the joint ICES/OSPAR Study Group on the Integrated Monitoring of Chemicals and their Effects (SGIMC). SGIMC was created specifically to provide the basis for advice to respond to the OSPAR request. SGIMC worked closely with the ICES Working Group on Biological Effects of Contaminants (WGBEC) and built upon work in preceding years by several other ICES groups, particularly the ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON), the Marine Chemistry Working Group (MCWG), and the Working Group on Marine Sediments in Relation to Pollution (WGMS).

The documents in this report were largely completed during the 2010 and 2011 meetings of SGIMC, and incorporated documents provided through WGBEC. They follow the established OSPAR structure for monitoring guidelines and associated background documents and technical annexes, as shown in Figure 1.1. Together with the background documents and technical annexes for chemical monitoring already adopted by OSPAR, this comprises a coherent basis for integrated chemical and biological effects monitoring. However, it should be noted that our knowledge regarding integrated monitoring and assessment will continue to evolve. In order to give some stability to assessments, it is important that future revisions of techniques and assessment criteria are harmonized with the MSFD cycle.

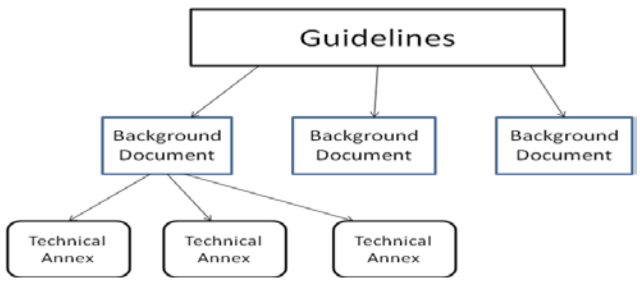

Figure 1.1. Structure of advice: guidelines provide concept and strategy, background documents provide description of available methodology and references, and technical annexes contain a detailed description of methods and advice on how to understand the measurements.

This report is a consolidation of the final advisory documents provided to OSPAR. The resultant availability of OSPAR background documents, assessment criteria, and quality assurance procedures for biological effects methods is summarized in Table 1.1. Documents not developed through SGIMC and, therefore, not included in this report are available through the OSPAR website (www.ospar.org). Parallel documents for sampling, chemical analysis of marine environmental materials (primarily fish, shellfish, and sediment), and assessment of monitoring data are also available on the OSPAR website.

Table 1.1. Biological effect techniques relevant to the ecosystem components for integrated monitoring and assessment of chemical and biological effects data. Status regarding availability of background documents, assessment criteria, and quality assurance. A: BEQUALM; B: between particular independent laboratories; C: QUASIMEME; D: BEAST; E: WGBEC; F: MED POL.

Biological effect technique	Background document	Assessment criteria	Quality assurance
Oyster and mussel embryo test	X	X	A
Sea urchin embryo test	X	X	B
Copepod test (*Tisbe*)	X	X	A
Whole-sediment bioassays	X	X	A
Sediment pore-water bioassays	X	X	A
Sediment seawater elutriates	X	X	A
DR-Luc	X	X	B (in future)
PAH metabolites	X	X	C, D
Cytochrome P450 1A activity (EROD)	X	X	A, B, F
Vitellogenin	X	X	E
Acetylcholinesterase	X	X	B, E
Comet assay	X	X	E
Micronucleus formation	X	X	B, F
DNA adducts	X	X	currently not available
Metallothionein	X	X	A (fish) F (mussels)
Lysosomal stability (cytochemical and neutral red)	X	X	B (fish) B, F (mussels)
Liver histopathology	X	X	A
Macroscopic liver neoplasms	X	X	A
Intersex in fish	X	X	B (in future)
Mussel histopathology (gametogenesis)	X	X	B (in future)
Imposex/intersex in gastropods	X	X	C
Stress on stress (SoS)	X	X	not required
Scope for growth	X	X	B
Externally visible fish diseases	X	X	A
Reproductive success in eelpout	X	X	A

BEQUALM, Biological Effects Quality Assurance in Monitoring Programmes, www.bequalm.org; QUASIMEME, organization that offers quality assurance for chemical endpoints, http://www.quasimeme.org; BEAST, BONUS+ Biological Effects of Anthropogenic Chemical STress: Tools for the assessment of Ecosystem Health project WGBEC, ICES Working Group on Biological Effects of Contaminants; MED POL, Mediterranean Pollution programme.

Currently, the background documents and assessment criteria are available for all biological effect techniques relevant to the ecosystem components for integrated monitoring and assessment of chemical and biological effects, apart from benthic fauna, sediment characteristics, and the use of passive samplers. These are important elements of the integrated scheme, and work to prepare background documents and assessment criteria needs to be undertaken as soon as possible.

We thank the various members of SGIMC and other groups who have been involved in this project over the years for their contributions to this report and the significant step forward that it represents for marine environmental monitoring and assessment. We acknowledge the very considerable efforts made by both the authors of the

various chapters and the other members of the ICES family who devoted many long days at (and between) expert group meetings to developing the conceptual framework that allowed such progress to be made, and then to the drafting and revision of the guidelines and supporting documents.

Participants in SGIMC meetings and associated workshops 2009–2011	Country
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2 Guidelines for the integrated monitoring and assessment of contaminants and their effects

Ian M. Davies, Matt Gubbins, Ketil Hylland, Thomas Maes, Concepción Martínez-Gómez, Colin Moffat, Thierry Burgeot, John Thain, and Dick Vethaak

2.1 General introduction

Our seas and oceans are dynamic and variable. They represent a fundamental component of global ecosystems and, as such, we need to be able to assess the health status of the marine environment. Furthermore, we need to be able to detect anthropogenically induced changes in seas and oceans and to identify the reasons for these changes. It is only through such understanding that we can advise on necessary and appropriate remedial responses, such as regulatory action, as well as report on any improvements resulting from OSPAR measures. There is a need to express clearly what is meant by the "health" of the marine environment, and for that purpose, we require indicators for the components of ecosystem health.

The marine environment receives inputs of hazardous substances through riverine inputs, direct discharges, and atmospheric deposition. The marine environment is the ultimate repository for complex mixtures of persistent chemicals. This means that organisms are exposed to a range of substances, many of which can cause metabolic disorders, an increase in disease prevalence, and, potentially, effects on populations through changes in, for example, growth, reproduction, and survival. There is general agreement that the best way to assess the environmental quality of the marine environment with respect to hazardous substances is to use a suite of chemical and biological measurements in an integrated fashion. In the past, monitoring to assess the "impact" of hazardous substances has been based primarily on measurements of concentration. This was because the questions being asked concerned concentrations of such substances in water, sediment, and biota, and such measurements were possible. However, in order to more fully assess the health of our maritime area, questions about the bioavailability of hazardous substances and their impact on marine organisms or processes are now being posed. Biological effects techniques have become increasingly important in recent years. The specific focus from OSPAR is on determining whether or not there are any unintended/unacceptable biological responses, or unintended/unacceptable levels of such responses, as a result of exposure to hazardous substances. Sometimes a biological response can be observed when the causative substance is below current chemical analytical detection limits; the development of imposex in gastropod molluscs as a result of tributyltin (TBT) is a case in point.

This guidance document is intended to complete the development of Joint Assessment and Monitoring Programme (JAMP) guidance for integrated monitoring of chemicals and their biological effects. The original JAMP guidelines for monitoring contaminants and biological effects in biota and sediments did not provide guidance for the optimum approach to monitoring or support the integrated assessment of concentrations and effects of contaminants across the OSPAR maritime area, although some contain references to supporting measurements (chemical, physical, and biological data) that aid the interpretation of monitoring data. Consequently, chemical analytical and biological effects data have usually been collected, reported, and assessed separately. Also, in some cases, the original guidelines do not provide guidance on the specific substances that should be determined in order to explicitly link concentrations and effects. An integrated approach to monitoring is based on the simultaneous measurement of contaminant concentrations (in biota, sediments, and, in some cases, water or passive samplers), biological effects parameters, and a range of physical and other chemical measurements so as to permit normalization and appropriate assessment.

Integrated monitoring of contaminants and their effects requires coordination of field sampling and sample-handling techniques, utilizing the same species/ population/individual for both types of measurement from the same area and sampled within the same time-frame. Furthermore, a set of supporting parameters should be measured at the same time, and such data have to be available for use in the final assessment, because biological effects may be influenced by factors such as temperature, stage of maturation, or size. Integration of effort in this way will yield additional information in a cost-effective manner, while also reducing the interannual variance of the data.

OSPAR has obligations to measure and monitor the quality of the marine environment and its compartments (water, sediments, and biota), the activities and inputs that can affect that quality, and the effects of those activities and inputs, and to assess what is happening in the marine environment as a basis for identifying priorities for action. OSPAR, together with HELCOM, have agreed on an ecosystem approach to managing the marine environment, under which OSPAR has committed to monitoring the ecosystems of the marine environment, in order to understand and assess the interactions between, and impact of, human activities on marine organisms. Integrated monitoring and assessment of contaminants in the marine environment and their effects will contribute effectively to the integrated assessment of the full range of human impacts on the quality status of the marine environment, as part of the ecosystem approach.

2.2 The OSPAR Hazardous Substances Strategy

The objective of the OSPAR Hazardous Substances Strategy (OSPAR Agreement 2003–2021) is to prevent pollution of the maritime area by continuously reducing discharges, emissions, and losses of hazardous substances, with the ultimate aim of achieving concentrations in the marine environment near background values for naturally occurring substances and close to zero for synthetic substances. The Hazardous Substances Strategy further declares that the Commission will implement this Strategy progressively by making every endeavour to move towards the target of the cessation of discharges, emissions, and losses of hazardous substances by the year 2020. In association with this and the other five OSPAR strategies, OSPAR has developed a Joint Assessment and Monitoring Programme (JAMP). This provides the basis for the monitoring activities undertaken by contracting parties to assess progress towards achieving OSPAR objectives. In relation to hazardous substances, the JAMP seeks to addresses the following questions:

- What are the concentrations of hazardous substances in the marine environment? Are those hazardous substances monitored at, or approaching, background levels for naturally occurring substances and close to zero for synthetic substances? How are the concentrations changing over time? Are the concentrations of either individual substances or mixtures of substances such that they are not giving rise to pollution effects?

- How can OSPAR's monitoring framework be improved and extended and better linked with the understanding of biological effects and ecological impacts of individual substances and the cumulative impacts of mixtures of substances?

There is a need to adopt an integrated approach to the monitoring of contaminants in the marine environment and the biological responses to the presence of hazardous substances. Such an approach would provide greater interpretative power in assessments of the state of the OSPAR maritime area with respect to hazardous substances and an improved assessment of progress towards achieving the objectives of the OSPAR Hazardous Substances Strategy.

2.3 EU Water Framework Directive and Marine Strategy Framework Directive

The marine environment is a precious heritage that must be protected, restored, and treated as such, with the ultimate aim of providing biologically diverse and dynamic oceans and seas that are safe, clean, healthy, and productive. It is in this context that the European Union has, over the last decade, developed its water policies so that now there is significant European legislation covering marine waters and the lakes and rivers that ultimately flow into our coastal ecosystems. The Water Framework Directive (Directive 2000/06/EC) establishes a framework for community action in the field of water policy, central to which is a good ecological status for water bodies. This is described on the basis of biological quality, hydromorphological quality, and physico-chemical quality. More recently, the European Union has implemented the Marine Strategy Framework Directive (Directive 2008/56/EC). At its heart is the concept of "Good Environmental Status" for all European waters and the provision of a framework for the protection and preservation of the marine environment, the prevention of its deterioration, and, where practicable, the restoration of that environment in areas where it has been adversely affected. "Good Environmental Status" (GES) will be assessed on a regional basis. The programmes of the various regional sea conventions, including OSPAR, will provide a valuable source of data for the assessments that will be required.

The Directive specifies that GES will be assessed against 11 qualitative descriptors. Descriptor 8 (Concentrations of contaminants are at levels not giving rise to pollution effects) has been interpreted as requiring assessments of contaminant concentrations and their biological effects.

A task group established by EC Joint Research Centre (JRC) interpreted this as meaning that the concentrations of contaminants should not exceed established quality standards (e.g. Environmental Quality Standards EQS, environmental assessment criteria (EAC)) and that the intensity of biological effects attributable to contaminants should not indicate harm at organism level or higher levels of organization. Commission Decision (2010/477/EU) noted that progress towards GES will depend on whether or not pollution is progressively being phased out (i.e. the presence of contaminants in the marine environment and their biological effects are kept within acceptable limits, so as to ensure that there are no significant impacts on or risk to the marine environment).

It is clear that assessment for Descriptor 8 will require both chemical and biological effects measurements. It is likely that a robust and holistic approach will seek to integrate the assessment of chemical and biological effects data into a single process.

2.4 Purpose of these guidelines

The purpose of this document is to provide guidance on integrated chemical and biological effects monitoring within the OSPAR area in the context of the Coordinated Environmental Monitoring Programme (CEMP) and the list of OSPAR priority chemicals. In addition, it provides a place for the associated technical annexes describing biological effects techniques, including a list of the supporting parameters that are required in an integrated programme, as well as the chemical determinands relevant to the effects being studied.

The guidelines are supported by associated background documents which provide information on the scientific background to the contaminants and biological effects measurements included in the programme, and on the derivations and values of the assessment criteria (background concentrations, background assessment concentrations, and environmental assessment criteria for chemical contaminants, and analogous assessment criteria for biological effects measurements).

2.5 Quantitative objectives; temporal trends and spatial programmes

The ultimate objectives of OSPAR monitoring activities relating to hazardous substances are to:

- assess the status (existing level of marine contamination and its effect) and trends of hazardous substances across the OSPAR maritime area;
- assess the effectiveness of measures taken for the reduction of marine contamination;
- assess harm (unintended/unacceptable biological responses) to living resources and marine life;
- identify areas of serious concern/hotspots and their underlying causes;
- identify unforeseen impacts and new areas of concern;
- create the background to develop predictions of expected effects and the verification thereof (hindcasting); and
- direct future monitoring programmes.

By being clear about the objective of the monitoring, the parameters for inclusion in the programme of work, the sampling strategy, methods of statistical analysis, and assessment methods can all be developed and specified. In the context of integrated monitoring, the planning aspect is crucial as it will ensure that operating procedures can be put in place that clearly detail all of the chemical, physical, and biological samples and data to be collected.

There is a need to perform monitoring to identify differences over time and across geographical space. This will be divided into two generic types:

- *spatial monitoring* to identify geographical variation within the OSPAR maritime area; and
- *temporal monitoring* aimed at identifying changes over time.

Although these two types of monitoring have been described separately, there is no reason why they cannot be carried out simultaneously, provided that this is incorporated into the design of the programme. The processes of integration for both these types of monitoring are closely related and hence should be developed simultaneously.

2.6 The integrated approach

The contribution made by an integrated programme involving both chemical and biological effects measurements is primarily that the combination of the different measurements increases the interpretive value of the individual measurements. For example, biological effects' measurements assist the assessment of the significance of measured concentrations of contaminants in biota or sediments. When biological effects measurements are carried out in combination with chemical measurements (or additional effects measurements), this provides an improved assessment allowing identification of the substances contributing to the observed effects. By bringing together monitoring disciplines that have tended to be conducted separately, an integrated assessment can improve our ability to explain the causes for hotspots detected during monitoring programmes. An integrated approach also has the advantage of combining and coordinating the various disciplines to achieve a greater understanding among those performing marine assessments of the contributions from the different components of a monitoring programme. This has the clear technical advantage that sampling of all relevant parameters at any particular sampling location will be assured. The economic benefit of an integrated approach comes from the fact that the samples and data are gathered during a single cruise and that the data can be directly compared/used with holistic assessment tools to provide truly integrated assessments.

The integration of sampling has four distinct connotations:

- sampling and analyses of same tissues and individuals;
- sampling of individuals for effects and chemical analyses from the same population as that used for disease and/or population structure determination at the same time;
- sampling of water, the water column (if included), and sediments at the same time and location as collecting biota; and
- simultaneous measurement of support parameters (e.g. hydrographic parameters) at any given sampling location.

Fundamental aspects of the design of an integrated programme include key environmental matrices (water, sediment, and biota), the selection of appropriate combinations of biological effects and chemical measurements, and the design of sampling programmes to allow the chemical concentrations, the biological effects data, and other supporting parameters to be combined for assessment. Some matrices/determinands are considered fundamental to the integrated assessment and are described as "core methods". Where additional matrices/determinands have been found to add value to the integrated assessment, these have been described as "additional methods" and are not considered essential. The basic structure of an integrated programme is illustrated in Figure 2.1.

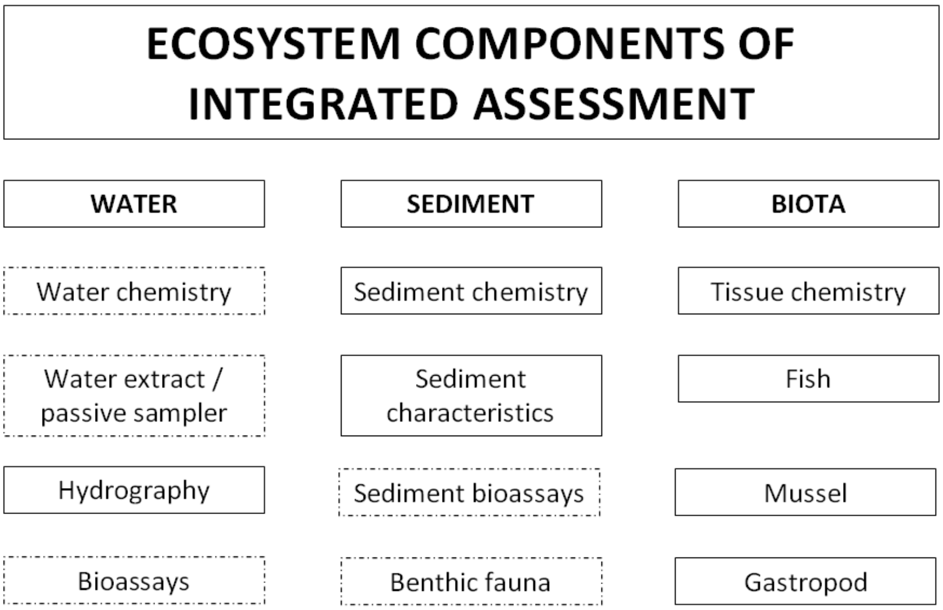

Figure 2.1. Overview of components in a framework for an integrated monitoring programme for chemical contaminants and their biological effects. Solid lines, core methods; broken lines, additional methods.

Chemical analyses to be included in an integrated programme for OSPAR purposes should cover the OSPAR priority hazardous substances. Analytical methods should be sufficiently sensitive to detect variation in environmental quality and should be supported by appropriate quality control and assurance. Biological effects methods to be included in an integrated programme have been identified by the ICES Working Group on the Biological Effects of Contaminants (WGBEC). They require the following characteristics:

- the ability to separate contaminant-related effects from influences caused by other factors (e.g. natural variability, food availability);
- sensitivity to contaminants (i.e. providing “early warning”);
- a suite of methods that covers a range of mechanisms of toxic action (e.g. oestrogenicity/androgenicity, carcinogenicity, genotoxicity, and mutagenicity); and
- the inclusion of at least one method that measures the “general health” of the organism.

Biological effects and chemical methods have been selected for the biota matrix (separated as fish and mussels) using these criteria. In addition, some physiological characteristics of individual fish are required, including gonadosomatic index (GSI), liver somatic index (LSI), and condition factor, as described in supporting technical annexes. Similarly, spawning status is relevant to mussel effect assessment. General designs for integrated monitoring of fish are presented in Figure 2.2 and of mussels in Figure 2.3. Designs for water, sediment, and gastropod monitoring are included as Figures 2.4, 2.5, and 2.6, respectively.

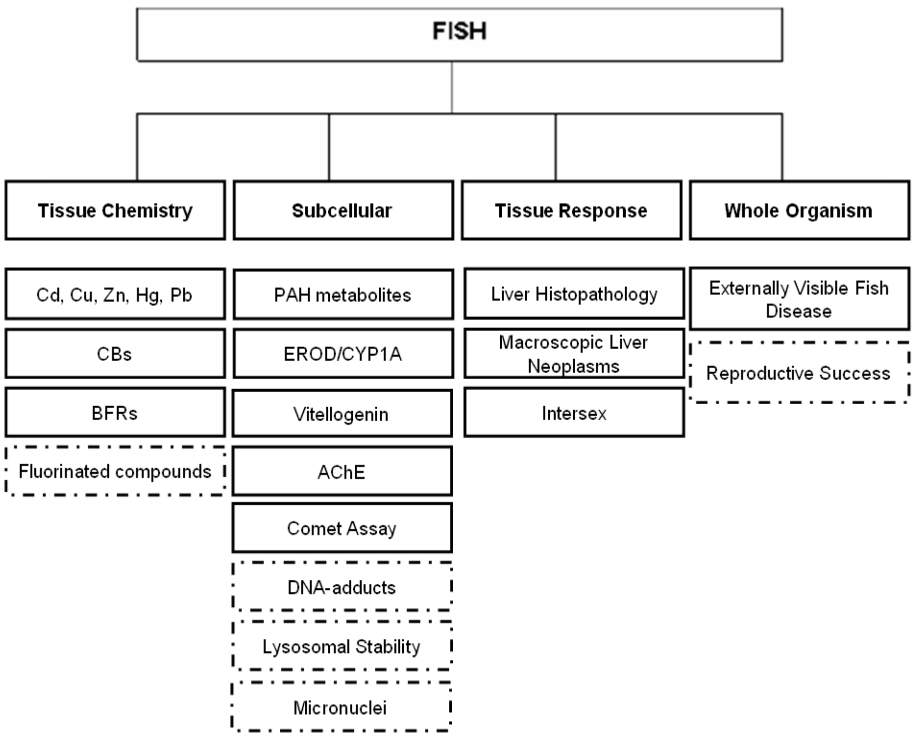

Figure 2.2. Methods included in the fish component of the integrated monitoring framework. Solid lines, core methods; broken lines, additional methods. CBs, chlorinated biphenyls; BFRs, brominated flame retardants; AChE, acetylcholinesterase.

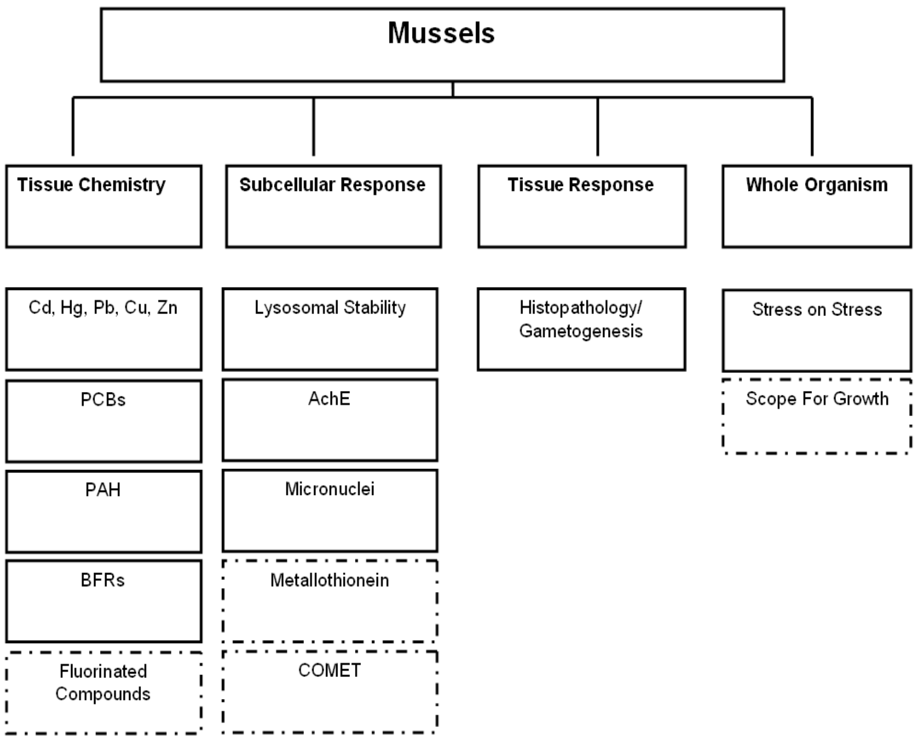

Figure 2.3. Methods included in the mussel component of the integrated monitoring framework. Solid lines, core methods; broken lines, additional methods. PCBs, polychlorinated biphenyls; PAH, polycyclic aromatic hydrocarbon; BFRs, brominated flame retardants; AChE, acetylcholinesterase.

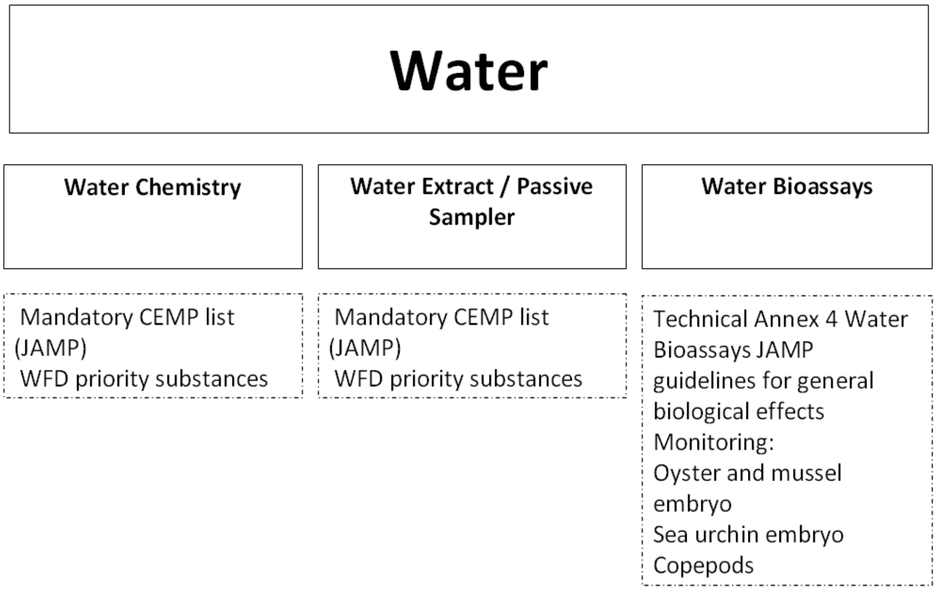

Figure 2.4. Methods included in the water component of the integrated monitoring framework. Solid lines, core methods; broken lines, additional methods.

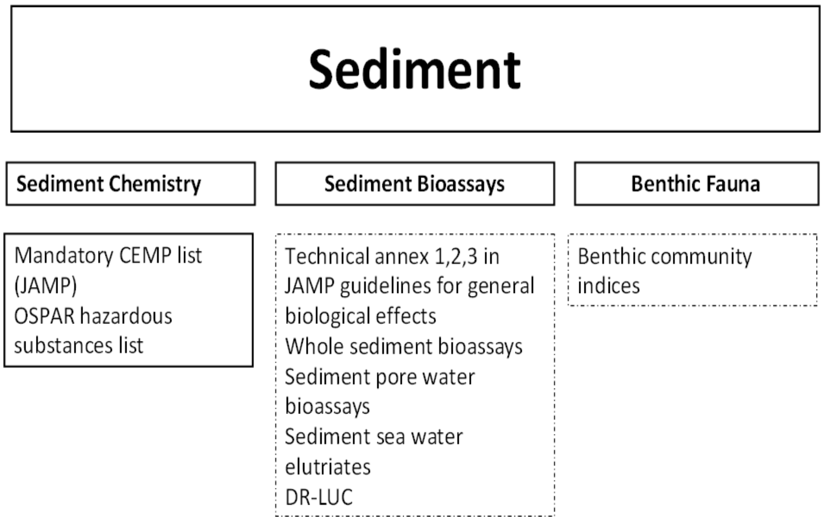

Figure 2.5. Methods included in the sediment component of the integrated monitoring framework. Solid lines, core methods; broken lines, additional methods.

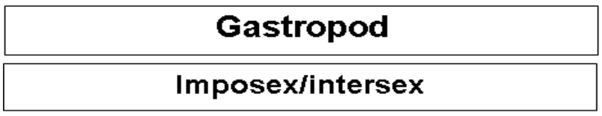

Figure 2.6. Methods included in the gastropod component of the integrated monitoring framework. Solid lines, core methods; broken lines, additional methods.

2.7 Sampling and analysis strategies for integrated fish and bivalve monitoring

The integration of contaminant and biological effects monitoring requires a strategy for sampling and analysis that includes:

- sampling and analyses of the same tissues and individuals;

- sampling of individuals for effects and chemical analyses from the same population as that used for disease and/or population structure determination at a common time;
- sampling of water, the water column, and sediments at the same time and location as collecting biota; and
- more or less simultaneous sampling for and determination of primary and support parameters (e.g. hydrographic parameters) at any given location.

Examples of sampling strategies for the integrated fish and shellfish schemes are shown in Figures 2.7 and 2.8. In order to integrate sediment, water chemistry, and associated bioassay components with the fish and bivalve schemes, sediment and water samples should be collected at the same time as fish/bivalve samples and from a site or sites that are representative of the defined station/sampling area.

Additional integrated sampling opportunities may arise from trawl/grab contents, for example, gastropods for imposex or benthos, and these should be exploited where possible/practicable.

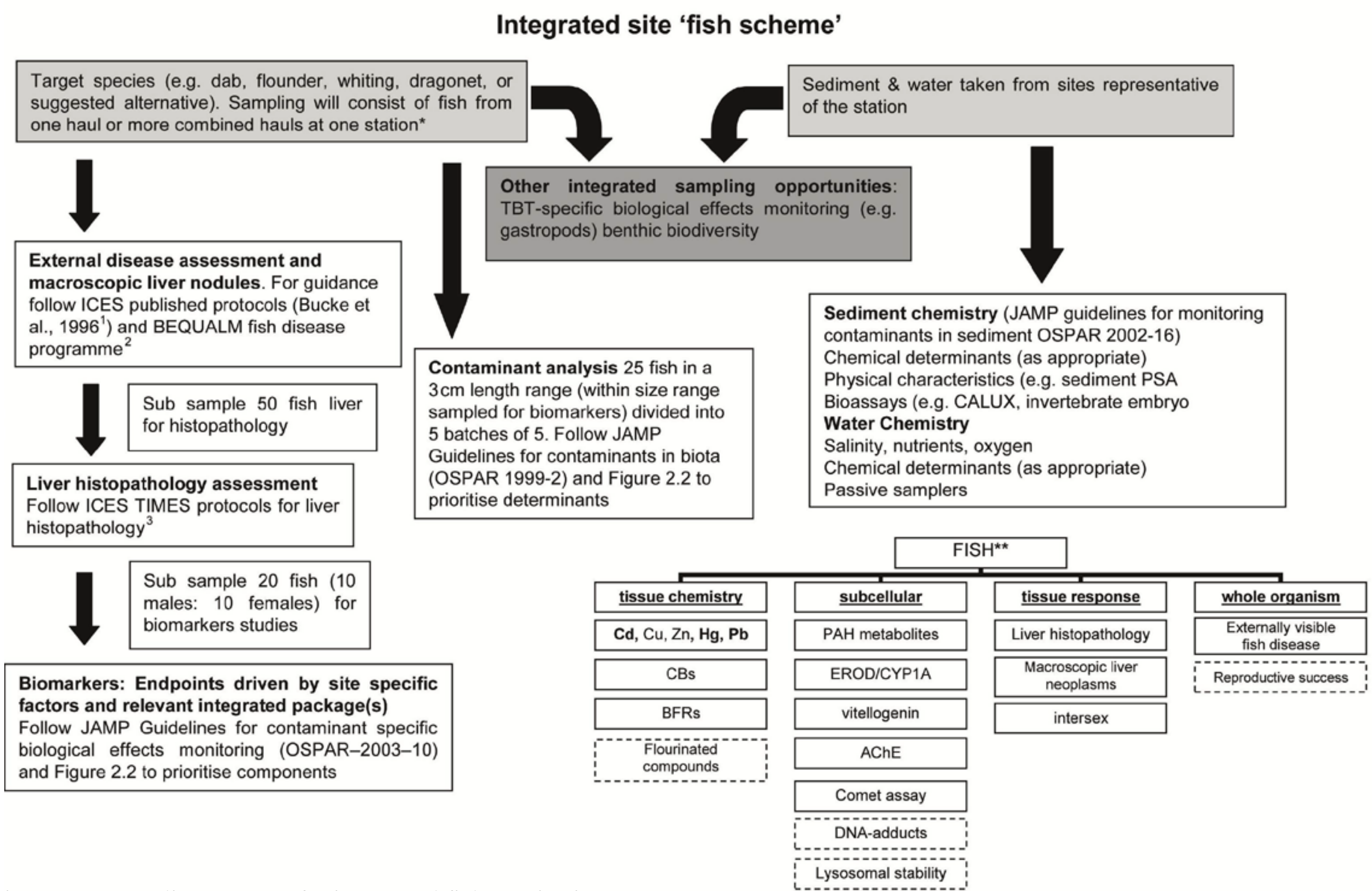

Figure 2.7. Sampling strategy for integrated fish monitoring.

**Figure 2.2 Overview of methods to be included in an integrated programme for selected fish species. (Solid lines – core methods, broken lines – additional methods).

1 Note: A station may be site specific or a larger defined area

2 Bucke, D., Vethaak, D., Lang, T., and Mellergaard, S. 1996. Common diseases and parasites of fish in the North Atlantic: training guide for identification. ICES Techniques in Marine Environmental Sciences, No. 27 pp.

3 BEQUALM: http://www.bequalm.org/fishdisease.htm

4 Feist, S. W., Lang, T., Stentiford, G. D. and Köhler A., 2004. The use of liver pathology of the European flatfish, dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring biological effects of contaminants. ICES Techniques in Marine Environmental Sciences, No. 38. 47 pp.

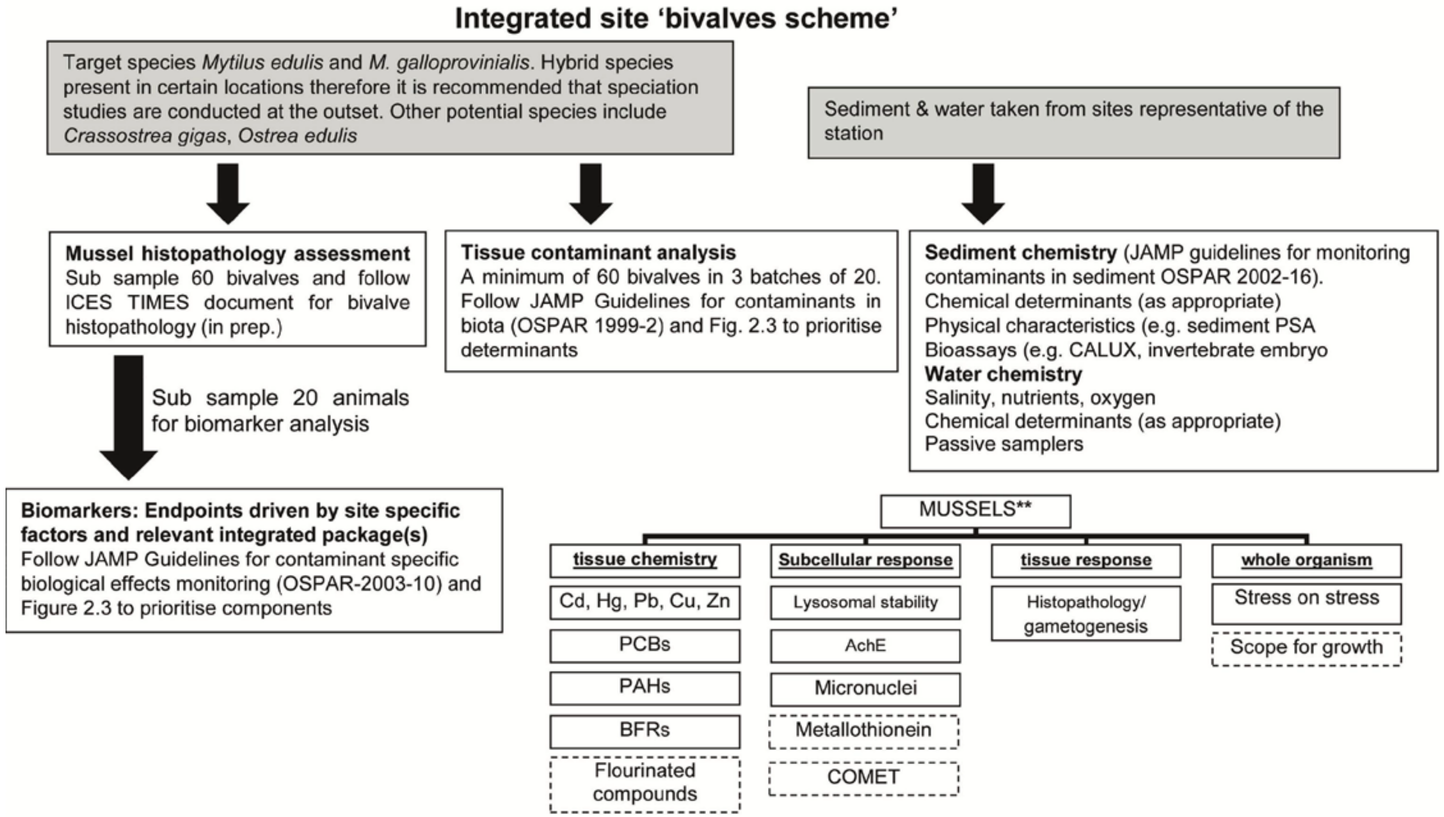

Figure 2.8. Sampling strategy for integrated bivalve monitoring.

** Figure 2.3 Overview of methods to be included in an integrated programme for selected bivalve species. (Solid lines – core methods, broken lines – additional methods).

2.8 The integrated assessment

It is not sufficient simply to coordinate sampling; integration must also involve a combined assessment of the monitored parameters, which must themselves be selected with the assessment aim in mind. Such a combined assessment may involve using environmental parameters as covariates in statistical analyses or they may be used to standardize effect-variables (e.g. temperature or seasonal effects on biomarker responses). Similarly, normalization procedures for the expression of contaminant concentrations in biota and sediment have been established. For example, defined bases (e.g. dry weight or lipid weight) are used for biota analyses, and sediment analyses are normalized to organic carbon or aluminium to minimize the influence of differences in bulk sediment properties. These procedures are described in detail in the OSPAR Co-ordinated Environmental Monitoring Programme (CEMP) Monitoring Manual (OSPAR, 2012).

Ultimately, the purpose of an integrated monitoring programme is to provide the necessary data to facilitate integrated assessments so that the status of the marine environment in relation to hazardous substances can be described as a contribution to general assessments of the quality status of the OSPAR maritime area (e.g. OSPAR Quality Status Reports – QSRs). In order to assess progress towards the objectives of the OSPAR Hazardous Substances Strategy, OSPAR has developed assessment criteria for contaminant concentration data. These are background concentrations (BCs), background assessment concentrations or criteria (BAC), and environmental assessment criteria (EAC). The use of these in data assessment, on both local and large (OSPAR Convention area) scales, is described in the CEMP Manual. The Manual also describes the statistical approaches to be used in comparing field data with assessment criteria to ensure rigorous and consistent assessments.

In the same way, OSPAR, with assistance from ICES, has more recently developed coherent sets of analogous assessment criteria for biological effects measurements. The concept of a background level of response is applicable to all effects measurements. Assessment criteria analogous to EAC (i.e. representing levels of response below which unacceptable responses at higher, e.g. organism or population, levels would not be expected) are applicable for some biological effects measurements, and these have been termed "biomarkers of effect". In other cases, the link to higher level effects is less clear, and these measurements have been termed "biomarkers of exposure", in that they indicate that exposure to hazardous substances has occurred. Importantly, the processes used to derive both BAC and their biological analogues and EAC and their analogues have been applied consistently to all chemical and effects measurements. The consequence is that the OSPAR objective of achieving background or near-background concentrations/effects represents targets based upon the same criteria across all parameters, and that EAC and their analogues represent similar levels of environmental risk. A table of the current assessment criteria for biological effects is presented in Section 30.

This coherence across the broad range of assessment criteria forms the basis for integrated assessment schemes. Progress towards the objectives of the Hazardous Substances Strategy was demonstrated in the QSR 2010 document, in that the status of all OSPAR priority contaminants could be presented in directly comparable "traffic light" formats (Figure 2.9).

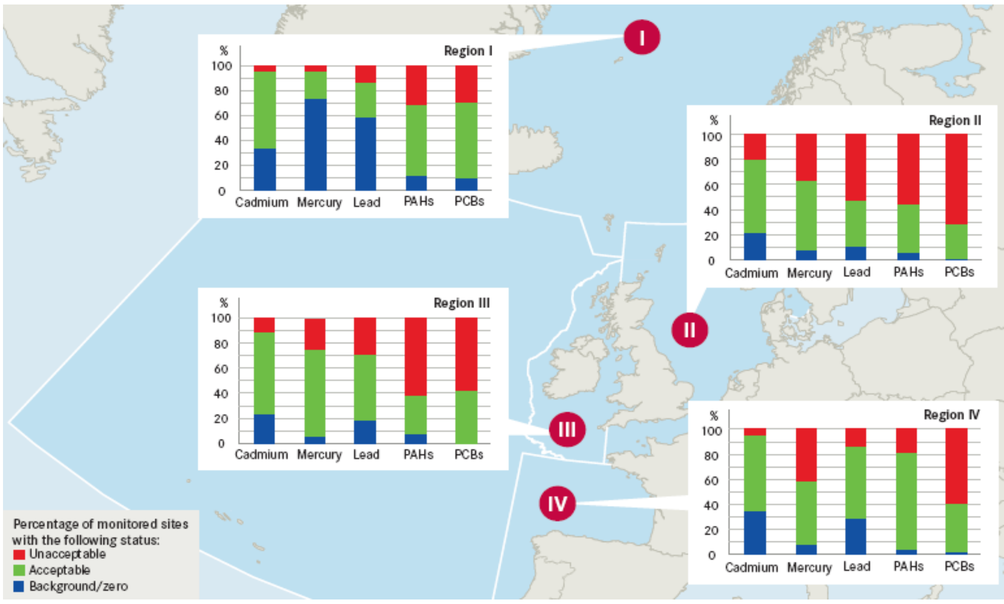

Figure 2.9. OSPAR regional-level integration of the concentrations of priority contaminants in fish, shellfish, and sediment from the OSPAR QSR 2010, Hazardous Substances chapter (OSPAR, 2010).

A comparable approach can be used in the assessment of biological effects data for which EAC and/or BAC have been developed. Furthermore, the coherence of assessment criteria across both chemical and biological effects measurements allows these two types of data to be brought together into a single integrated assessment scheme. The "traffic light" presentation is equally applicable to biological effects data and can be used to present data integrated on a range of geographical scales from the single sampling site to the regional scale, as required under MSFD. The application of this approach is described in Section 30.

3 Background document: polycyclic aromatic hydrocarbon metabolites in fish bile

Ketil Hylland, Dick Vethaak, and Ian M. Davies

3.1 Background

Analyses of polycyclic aromatic hydrocarbon (PAH) metabolites in fish bile have been used since the early 1980s as a biomarker of exposure to PAH contamination. The presence of metabolites in bile (and in urine) is the final stage of the biotransformation process through which lipophilic compounds are transformed into a more soluble form and then passed from the organism in bile or urine.

As a biomarker of exposure, measuring PAH metabolites in bile has many advantages over other techniques that require sophisticated tissue-preparation protocols. The pretreatment of bile samples requires relatively simple dilution steps prior to analysis by direct fluorescence measurement. The bile is diluted in methanol:distilled water (1:1), and fluorescence is measured with a fluorometer. Fixed-wavelength fluorescence is a suitable screening method for these samples, whereas HPLC/F or GC-MS selected ion monitoring (SIM) is utilized for qualitative and quantitative measures (Lin *et al.*, 1996; Aas *et al.*, 2000a,b; Jonsson *et al.*, 2003; Ariese *et al.*, 2005).

Bile is generally stored in the gall bladder prior to episodic release into the oesophagus, where bile salts have a function as part of the digestive process. This period of storage permits a degree of accumulation of metabolites and hence an increase in their concentration. The periodic release of bile does, however, introduce a variable into the technique that must be accounted for. The feeding status of fish has been demonstrated to influence both the volume and the density of the bile (Collier and Varanasi, 1991).

The ability of fish to biotransform PAHs into less lipophilic derivatives means that reliance on the detection of parent PAHs alone may lead to an underestimation of the *in vivo* exposure level of PAHs in the fish. PAH metabolite detection, on the other hand, represents a quantification of the flux of PAHs streaming through the fish's body. From a toxicological point of view, flux information is more relevant to estimating the actual biotic stress caused by PAH exposure than the body burden data of the unmetabolized parent PAH compounds in tissues (most often liver). Despite this, body burden measurements are still more commonly used within monitoring studies than metabolite determination.

3.2 Dose–response (species-specific)

The PAH compounds are metabolized rapidly in organisms, and it is the endpoint of this metabolism that is measured in the bile using chemical analysis. A consistent dose–response relationship has been demonstrated in laboratory studies between PAH exposure and the subsequent presence of metabolites in bile (Beyer *et al.*, 1997; Aas *et al.*, 2000a). To establish a good dose–response relationship in field studies, it is necessary to focus on aspects that influence the excretion of bile.

The method requires that bile is available in the gall bladder. Because fish renew bile as part of normal metabolism and excrete it during digestion, it is important to know about the dietary status of the organism to establish a dose–response relationship. If the fish have fed just before sampling, the gall bladder may be more or less empty. After the gall bladder has been emptied, it will fill up, and metabolites will be concentrated up to a plateau level corresponding to the exposure regime. Consequently, the time since last feeding is important for the dose–response relationship. Fish generally have very efficient metabolic excretion of most PAHs, and it has been demonstrated that most of the PAHs will be excreted 2–8 d following exposure. This means that the PAH metabolites determined in bile will represent exposures on the scale of days and, at most, 2 wk.

It has been demonstrated in several field and laboratory studies that there is a good correlation between PAH exposure and bile metabolites. Because of the rapid metabolism and the correlation between bile content and digestive status, it is difficult to form a dose–response relationship that can be used to quantify exposure. Work has been done to try to correlate bile metabolite concentration to digestive status by correlating it to the amount of protein or biliverdin in the bile. Absorbance at 380 nm is also used (similar to biliverdin; K. Hylland, pers. comm.). This normalization is not standardized because it has been shown to only explain part of the variability, but it is recommended to be included in the interpretation of results. In laboratory studies it is normal to stop feeding the fish some days before sampling to ensure the bile quality. In field sampling, this can be taken into account by holding the fish for some days in tanks before sampling, although this has some logistical challenges.

3.3 Species sensitivity

The background level differs between species, so it is important to establish a good baseline before using new species. It may be expected that species with fatty livers (i.e. most gadoids) may metabolize PAHs more slowly as more will partition into fat, but this has not been documented experimentally. Species differences have, in general, to be considered when calculating BAC and EAC, although in some cases the resulting assessment criteria are so similar that combined criteria for several species are justified (Table 3.1 gives an example).

3.4 Relevance of other factors

As mentioned above, food availability will affect the concentration of PAH metabolites in bile. In an assessment of data for more than 500 individual Atlantic cod (*Gadus morhua*) sampled over five years of national monitoring, variables such as size/age and sex explained some variability in multiple regression models (Ruus *et al.*, 2003). This could be the result of different feeding preferences, but also endogenous processes. In addition, the fat content of the liver (measured as liver somatic index, LSI) came out as significant, presumably because fat decreases the availability of PAHs to the cellular compartments of liver cells. There are indications of seasonal differences between summer and winter values of PAH metabolites in dab (*Limanda limanda;* Kammann, 2007).

3.5 Background responses

Baseline levels of PAH metabolites have been established for many of the species relevant to monitoring in Norwegian coastal and offshore waters. From Ruus *et al.* (2003), values for the relevant species are: (all values standardized to absorbance at 380 nm) Atlantic cod: 0.6–4 μg kg^{-1} bile, flounder (*Platichthys flesus*) 27–89 μg kg^{-1} bile, dab 3.1–34 μg kg^{-1} bile, plaice (*Pleuronectes platessa*) 0.4–3 μg kg^{-1} bile (all quantified using HPLC separation and fluorescence detection and quantification). Standardization at 380 nm is used to remove variability caused by bile salts.

3.6 Assessment criteria

Assessment criteria for PAH metabolites such as BAC have been derived from reference sites (Table 3.1). EAC can be derived from toxicological experiment data by linking oil exposure and PAH metabolites in fish with DNA adducts and fitness data (Skadsheim, 2004; Skadsheim *et al.*, 2009), where the latter serves as the effect quantity for the calculation of the EAC presented in Table 3.1. Some variation in PAH metabolites in bile appear to be related to sex and size/age (Ruus *et al.*, 2003), knowledge of which should be included in the sampling design.

3.7 Quality assurance

A general protocol outlining analytical strategies and their strengths as well as weaknesses has recently become available (Ariese *et al.*, 2005). International intercalibration exercises for the determination of PAH metabolites in fish bile have been carried out in collaboration between an EU project and QUASIMEME.[1] Reference bile samples were generated and are now available through IRMM, JRC, Geel, Belgium (http://www.irmm.jrc.be/html/homepage.html). An intercalibration for PAH metabolites also took place in the framework of the EU-funded BONUS project "BEAST" in 2010.

3.8 Acknowledgement

This review was derived from an overview (Tables 3.2 and 3.3) prepared for the Norwegian offshore companies through OLF (Hylland *et al.*, 2006a).

1 QUASIMEME: organization that offers quality assurance for chemical endpoints; http://www.quasimeme.org

Table 3.1. Biological assessment criteria (BAC) and environmental assessment criteria (EAC) for two PAH metabolites, different fish species, and methods. Data partly taken from ICES (2009a)

Biological effect	Fish species	BAC [µg ml⁻¹] HPLC-F	EAC [ng g⁻¹] GC-MS
Bile metabolite 1-hydroxypyrene	Dab	16	
	Cod	21	483
	Flounder	16 [d]	
	Haddock	13	
	Dab, cod, haddock	17	
	Turbot		909
	Halibut		745
Bile metabolite 1-hydroxyphenanthrene	Dab	3,7	
	Cod	2.7	518
	Flounder	3.7 [d]	
	Haddock	0.8	
	Dab, cod, haddock	2.4	
	Turbot		1 832
	Halibut		262
		BAC [µg ml⁻¹] synchronous fluorescence 341/383 nm	**EAC [µg ml⁻¹] Fixed wavelength fluorescence 341/383 nm**
Bile metabolites of pyrene-type	Dab	0.15	22 [a]
	Cod	1.1	35
	Flounder	1.3	29 [b]
	Haddock	1.9	35 [c]
	Turbot		29
	Halibut		22
	Herring/sprat		16

Assessment criteria based on [a]halibut, [b]turbot, [c]cod, and [d]dab.

Table 3.2. Overview of field and laboratory studies—PAH metabolites measured by fixed fluorescence (Hylland *et al.*, 2006a)

Species	Substance (lab/field)	Test concentrations/area	Exposure time	Metabolite	Baseline	Control or reference	exposed /control
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline				
Cod (*Gadus morhua*)	Feral fish	Egersund	Baseline non-polluted area	Naph type Pyren type BaP type	5.3 µg ml^{-1} 0.8 µg ml^{-1} 0.4 µg ml^{-1}		
Cod (*Gadus morhua*)	Feral fish	Sleipner	Baseline polluted area	Naph type Pyren type BaP type	6.1 µg ml^{-1} 1.0 µg ml^{-1} 0.5 µg ml^{-1}		
Cod (*Gadus morhua*)	Feral fish	Statfjord	Baseline polluted area	Naph type Pyren type BaP type	5.9 µg ml^{-1} 0.9 µg ml^{-1} 0.3 µg ml^{-1}		
Cod (*Gadus morhua*)	Feral fish	Frøy, ceased installation 10 000 m (ref) 2 000–200 m	Baseline polluted area	Naph type Pyren type BaP type		3.9 µg ml^{-1} 0.6 µg ml^{-1} 0.3 µg ml^{-1}	1.1–1.1 1.1–0.9 0.9–0.9
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph type Pyren type BaP type	2.15 µg g^{-1} 1.63 µg g^{-1} 0.69 µg g^{-1}		
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph type Pyren type BaP type	5.8 µg g^{-1} 1.7 µg g^{-1} 0.8 µg g^{-1}		
Cod (*Gadus morhua*)	Laboratory	1 ppm crude oil Statfjord B	14 d				
Cod (*Gadus morhua*)	Laboratory	0.06–0.25–1 ppm oil	Average 3, 7, 14, 24 d	Naph type Pyren type BaP type		3.9 µg g^{-1} 2.6 µg g^{-1} 1.0 µg g^{-1}	7,5–23,7–31,4 3,6–10,6–13 1,7–2,4–2,2
Cod (*Gadus morhua*)	Laboratory	0.06–0.25–1 ppm oil	Average 3, 17, 31 d	Naph type Pyren type BaP type		53.1 µg g^{-1} 7.0 µg g^{-1} 1.0 µg g^{-1}	0.7–2.3–2.9 1–2.9–3.3 1.1–1.5–1.5
Cod (*Gadus morhua*)	Laboratory	Oil 0.06–0.25–1 ppm	30 d	Naph type Pyren type BaP type		7.1 fi 2 fi 0.8 fi	5.1–9.5–227.5 6.4–12.7–43.3 2.3–3.6–9.6
Cod(*Gadus morhua*)	Laboratory	PW Oseberg, 1:1 000–1:200–0.2 ppm oil - 0.2 ppm oil + PAH mix	15 d	Naph type Pyren type, BaP type		12.6 µg ml^{-1} 4 µg ml^{-1} 1.8 µg ml^{-1}	1.3–2.5–3.6–5.4 1.7–3.7–4.1–17.8 1.3–1.8–1.5–2.4
Cod (*Gadus morhua*)	Field, Caged	North Sea–Statfjord, 10 000–2 000–500 m German Bight G	5.5 wk	Naph type Pyren type BaP type	7.5 µg ml^{-1} 3.1 µg ml^{-1} 1.2 µg ml^{-1}	0,7 0,7 0,8	1.7–1.9–2.1 1.2–1.5–1.6 1.2–1.1–1.2

Cod (*Gadus morhua*)	Field, Caged	German Bight G4 (ref) G1–G2–G3	5.5 wk	Naph type	7.5 µg ml^{-1}	0,4	0.9–0.9–1.6
				Pyren type	3.1 µg ml^{-1}	0,5	0.8–0.9–1.7
				BaP type	1.2 µg ml^{-1}	0,7	0.8–1–1.3
Cod (*Gadus morhua*)	Field, Caged	North Sea–Troll, 1 000–500 m	6 wk	Naph type	4.6 µg ml^{-1}	1,4	1.7–2.5
				Pyren type	2.4 µg ml^{-1}	0,9	1.1–1.3
				BaP type	0.9 µg ml^{-1}	1,1	1.1–1.3
Cod (*Gadus morhua*)	Field, Caged	North Sea–Tampen, 10 000–2 500–1 000–500 m	6 wk	Naph type		8.8 µg ml^{-1}	1.0–1.5–1.2–1.2
				Pyren type		1.4 µg ml^{-1}	0.9–0.7–0.8–0.9
				BaP type			
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Egersund	Baseline non-polluted area	Naph type	5.1 µg ml^{-1}		
				Pyren type	1.4 µg ml^{-1}		
				BaP type	0.7 µg ml^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Sleipner	Baseline polluted area	Naph type	6.8 µg ml^{-1}		
				Pyren type	1.9 µg ml^{-1}		
				BaP type	0.8 µg ml^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Statfjord	Baseline polluted area	Naph type	11.2 µg ml^{-1}		
				Pyren type	2.5 µg ml^{-1}		
				BaP type	0.7 µg ml^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents Sea		Naph type	2.52 ug g^{-1}		
				Pyren type	1.69 ug g^{-1}		
				BaP type	0.77 ug g^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents Sea		Naph type	2.0 ug g^{-1}		
				Pyren type	1.3 ug g^{-1}		
				BaP type	0.6 ug g^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Frøy, ceased installation 10 000 m (ref) 2 000–200 m	Baseline polluted area	Naph type		5.6 µg ml^{-1}	1.3–2.2
				Pyren type		1.4 µg ml^{-1}	1.4–0.7
				BaP type		0.75 µg ml^{-1}	1.8–0.6
Sheepshead minnow (*Cyprinodon variegatus*)	Laboratory	North Sea oil A 0.1–0.4–0.7 ppm	5 wk	Naph type		6 916	2.3–6.2–9.3
				Pyren type		569	2.5–5–6.3
				BaP type		107	4–13.1–19.2
Sheepshead minnow (*Cyprinodon variegatus*)	Laboratory	North Sea oil B 0.1–0.9–5.6 ppm	6 wk	Naph type		18 164	1.8–4.3–12.5
				Pyren type		438	5.6–12.6–30.8
				BaP type		110	12.6–42.7–123.9
Sheepshead minnow (*Cyprinodon variegatus*)	Laboratory	2–14–214 ppb	5 wk	Naph type		267 280	0.9–2.2–18.6
				Pyren type		9 926	0.9–1.5–9.6
				BaP type		5 152.7	3–17.4–207
Polar cod (*Boreogadus saida*)	Laboratory, feral fish 2001, 2002	1.5 ppm Statfjord A oil , baseline, control	14 d	Naph type	16.0 ug g^{-1}	2	16.9
				Pyren type	0.9 ug g^{-1}	5,5	74.4
				BaP type	0 ug g^{-1}	0	1.8

Table 3.3. PAH metabolites in marine fish—measured by GC-MS (Hylland *et al.*, 2006a)

Species	Substance (lab/field)	Test concentrations	Exposure time	Metabolite	Baseline	Control or reference	Exposed/control
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph sum	150.6 ng g^{-1}		
				Phen sum	61.2 ng g^{-1}		
				Pyren	4.6 ng g^{-1}		
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph sum	1 285 ng g^{-1}		
				Phen sum	220 ng g^{-1}		
				Pyren	3.5 ng g^{-1}		
Cod (*Gadus morhua*)	Feral fish	Egersund	Baseline non-polluted area	Naph sum	2 005.1 ng g^{-1}		
				Phen sum	230.2 ng g^{-1}		
				Pyren	3.9 ng g^{-1}		
Cod (*Gadus morhua*)	Feral fish	Sleipner	Baseline polluted area	Naph sum	1 296.1 ng g^{-1}		
				Phen sum	197.8 ng g^{-1}		
				Pyren	0		
Cod (*Gadus morhua*)	Feral fish	Statfjord	Baseline polluted area	Naph sum	1 361.7 ng g^{-1}		
				Phen sum	351.1 ng g^{-1}		
				Pyren	4.0 ng g^{-1}		
Cod (*Gadus morhua*)	Laboratory	0.06–0.25–1 ppm oil	Average 3, 7, 14, 24 d	Naph sum		2 549 ng g^{-1}	4.6–13.4–23.6
				Phen sum		691 ng g^{-1}	7.7–22.9–34.9
				Pyren		27 ng g^{-1}	7.3–16.2–25.1
Cod (*Gadus morhua*)	Laboratory	0.06–0.25–1 ppm oil	Average 3, 17, 31 d	Naph sum		5 702 ng g^{-1}	4–13.3–12.7
				Phen sum		377 ng g^{-1}	10.5–40.3–48.7
				Pyren		5 ng g^{-1}	8.6–63–88.4
Cod (*Gadus morhua*)	Field, caged	North Sea—Statfjord, 500–2 000–10 000 m		Naph sum		1 150 ng g^{-1}	3.0–2.0–1.3
				Phen sum		340 ng g^{-1}	3.5–2.7–2.5
				Pyren			
Cod (*Gadus morhua*)	Field, caged	North Sea—Troll, 1 000–500 m	6 wk	Naph sum	1 515.1 ng g^{-1}	1.1	1.1–1.2
				Phen sum	327.2 ng g^{-1}	1.6	2.1–2.0
				Pyren	173.2 ng g^{-1}	1.2	0.9–1.2
Cod (*Gadus morhua*)	Field, caged	North Sea—Tampen, 10 000–2 500–1 000–500 m	6 wk	Naph sum		965.3 ng g^{-1}	0.9–1.7–0.9–1
				Phen sum		934.5 ng g^{-1}	1.4–3–1.8–1.5
				Pyren		3.7 ng g^{-1}	0–0–0.5–0.0
Cod (*Gadus morhua*)	Field, caged	North Sea—Statfjord, 10 000–2 000–500 m	5.5 wk	Naph sum	228 ng g^{-1}	0.2	0.9–1.1–0.9
				Phen sum	482 ng g^{-1}	2.0	3–4.5–6.7
				Pyren	28 ng g^{-1}	10.2	29.5–31.1–41.5
Cod (*Gadus morhua*)	Field, caged	German Bight G4 (ref) G1–G2–G3	5.5 wk	Naph sum	228 ng g^{-1}	0.8	1–1–1.9
				Phen sum	482 ng g^{-1}	1.0	0.7–0.8–0.8

				Pyren	28 ng g^{-1}	0.0	0–0–0
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Egersund	Baseline non-polluted area	Naph sum	1 346.9 ng g^{-1}		
				Phen sum	526.8 ng g^{-1}		
				Pyren	5.7 ng g^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Sleipner	Baseline polluted area	Naph sum	1 111.5 ng g^{-1}		
				Phen sum	331.5 ng g^{-1}		
				Pyren	10.4 ng g^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Statfjord	Baseline polluted area	Naph sum	1 279.7 ng g^{-1}		
				Phen sum	331.9 ng g^{-1}		
				Pyren	3.1 ng g^{-1}		
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents Sea		Naph sum	1 474 ng g^{-1}		
				Phen sum	165 ng g^{-1}		
				Pyren	0		
Polar cod (*Boreogadus saida*)	Laboratory, feral fish 2001, 2002	1.5 ppm Statfjord A oil, baseline, control	14 d	Naph sum	1 330 ng g^{-1}	1.3	114
				Phen sum	538 ng g^{-1}	0.9	90
				Pyren	52 ng g^{-1}	14.6	60

4 Background document: cytochrome P450 1A activity (EROD)

Ketil Hylland, Thomas Maes, Concepción Martínez-Gómez, Ulrike Kamman, Matt Gubbins, and Ian M. Davies

4.1 Introduction

The cytochrome P450 1A family of enzymes is responsible for the primary metabolism of planar polyaromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) and the activation of several procarcinogens, such as benzo[*a*]pyrene. 7-Ethoxyresorufin is a convenient artificial substrate which was developed as a safe, sensitive assay by Burke and Mayer (1974). Thus, the term "EROD" has been adopted as a measure of CYP1A activity in aquatic organisms (Stagg and McIntosh, 1998).

In addition to being substrates for biotransformation, planar compounds such as PAHs, PCBs, and dioxins also induce synthesis of cytochrome P450 1A by binding to the cytosolic aryl hydrocarbon (Ah) receptor/ARNT complex. Measurement of EROD activity is the tool used currently to quantify this induction. The induction of cytochrome P450 enzymes in fish liver was first suggested as an indicator of environmental contamination in the 1970s by Payne (1976), and has now gained widespread use (see, e.g., Förlin and Haux, 1990; Goksøyr *et al*., 1991a; George *et al*., 1995a; Whyte *et al*., 2000) and been standardized by ring-testing (BEQUALM, 2000).

4.2 Dose–response

In a review, Whyte *et al*. (2000) rank chemicals according to the level of EROD activity they induce in treated or exposed fish when compared with untreated or control fish. Contaminants that induce EROD less than tenfold above control levels are considered "weak" inducers, 10- to 100-fold are "moderate" inducers, and chemicals that elicit >100-fold induction are considered "strong" inducers. Dioxins, planar PCBs, and PAHs (benzo[*a*]pyrene) are categorized as "strong" inducers. Over 25 studies have observed induction of hepatic EROD by benzo[*a*]pyrene in 15 species of fish (Whyte *et al*., 2000).

4.3 Relevance of other factors

Several endogenous and exogenous factors have been shown to affect hepatic EROD. The most important endogenous factors for most fish species are gender, reproductive status, and season, all of which can be controlled through sampling design. In addition, environmental temperature has been shown to affect EROD (Sleiderink *et al*., 1995a; Lange *et al*., 1999). Seasonal cycles in EROD induction have been observed for rainbow trout (*Oncorhynchus mykiss*; Förlin and Haux, 1990), flounder (*Platichthys flesus*; von Westernhagen *et al*., 1981; Hylland *et al*., 1996), plaice (*Pleuronectes platessa*; George and Young, 1986) and salmon (*Salmo salar*; Larsen *et al*., 1992), most likely owing to changes in both water temperature and reproductive cycles (which it is not really possible to separate in the field). The main age-related factors are time of exposure/accumulation, food selection, and reproductive stage.

Several species have baseline EROD activities within the same order of magnitude among different studies/measurements and also show greater than tenfold EROD induction after contaminant exposure (Whyte *et al.*, 2000). These are, however, mostly freshwater species.

CYP1A expression is suppressed in spawning females because of interference of oestrogens (e.g. 17β-oestradiol, E2, or xenoestrogen) with transcription of the gene. This may also lead to an underestimation of a PAH-type response of EROD activity; however, this hormone also controls the induction of vitellogenin (Vtg; egg-yolk protein), which is produced by the liver during gonadal recrudescence. Therefore, interference of CYP1A induction by environmental oestrogens can be assessed.

Dietary factors may be potentially important for the induction of CYP1A. First, aryl hydrocarbon receptor (AhR) ligands may be ingested by the organism in the food. Second, proper nutrition is a prerequisite for enzyme systems to function properly. Hylland *et al*. (1996) reported an elimination of the EROD response (i.e. to control levels) in benzo[*a*]pyrene-treated flounder deprived of food for one month.

4.4 Background responses

Baseline levels of EROD in seven marine species have been estimated from results derived from the joint ICES/OSPAR WKIMON III meeting (ICES, 2007a) and recent data submitted to the ICES database (Table 4.1). The fish were from sites that the contracting parties consider to be reference stations (i.e. no known local sources of contamination) or those areas not considered unequivocally as reference sites but considered to be less affected by human and industrial activity. The datasets from which these values have been derived are described in Table 4.2. Further information on the baseline levels and dose–response of EROD activity in experimental systems and field studies is given in Tables 4.3 and 4.4.

4.5 Assessment criteria

Background response ranges have been developed as described above, and 90th percentiles of values from reference sites can be used to distinguish between ″background″ and ″elevated″ responses. Because many factors are known to influence EROD activity (see above), and because it is difficult to correct for all in the assessment of data, it is advisable to include an appropriate reference group in studies that include EROD as an endpoint. The information provided in Table 4.2 will also allow data to be assessed against the appropriate assessment criteria for fish species, gender, size, sampling season, and bottom-water temperature.

4.6 Quality assurance

Cytochrome P450 1A is possibly the most widely used biomarker. There have been three international intercalibrations for the method, all within BEQUALM. The intercalibrations have pinpointed variability relating to most steps in the analytical process, except possibly the enzyme kinetic analysis itself. It is imperative that laboratories have internal quality assurance procedures (e.g. use internal references samples with all batches of analyses).

4.7 Acknowledgement

The current review has been derived from an overview prepared for the Norwegian offshore companies through OLF (Hylland *et al.*, 2006a), the joint workshop ICES/OSPAR WKIMON III (ICES, 2007a), and the workshop SGIMC (ICES, 2009a).

Table 4.1. EROD assessment criteria in fish target species used in biomonitoring programmes around European waters. EROD background responses established are restricted to the sampling conditions and the length of the specimens used. The values of the assessment criteria must be considered as provisional and should be updated and revised when more data become available

EROD assessment criteria S9 fraction	Sampling season	Bottom-water temperature range (° C)	length (cm)	Sex	Background response range EROD activity (pmol min^{-1} mg^{-1} protein) 90P	Elevated response range EROD activity (pmol min^{-1} mg^{-1} protein) 90P	*N*
Dab (*Limanda limanda*)	August–November	10–18	12–25	Females	≤178	>178	556
				Males	≤147	>147	571
European flounder (*Platichthys flesus*)	August–November	10–18	20–25	Females and/or males	≤24	>24	65
Plaice (*Pleuronectes platessa*)	January	5–10	18.5–22.5	Males	≤10	>10	116
EROD assessment criteria microsomal fraction						**Elevated Response Range EROD activity (pmol min^{-1} mg^{-1} prot) 90P**	***n***
Dab (*Limanda limanda*)	August–November	10–18	20–30	Females and/or males	≤780	>780	53
Cod (*Gadus morhua*)	August–November	10–18	30–45	Females and/or males	≤145	>145	198
Plaice (*Pleuronectes platessa*)	September	7–10	40–60	Females and/or males	≤255	>255	64
Four spotted megrim (*Lepidorhombus boscii*)	September–October	11.7–12.7	18–22	Females and/or males	≤13	>13	317
Dragonet (*Callionymus lyra*)	September–October	12.0–12.8	15–22	Females and/or males	≤202	>202	159
Red mullet (*Mullus barbatus*)	April	13.3–15.3	12–18	Males	≤208	>208	40

Table 4.2. Description of data used in setting background and elevated response ranges

EROD assessment criteria	Sampling season	Bottom-water temperature range (° C)	Length (cm)	Sex	EROD background response activity median (pmol min^{-1} mg^{-1} protein)	Upper limit of EROD background response activity P90 (pmol min^{-1} mg^{-1} protein)	*N*
Dab (*Limanda limanda*)	August–November	10–18	12–25	Females and/or males	<30 †	<152 †	1 034
European flounder (*Platichthys flesus*)	August–November	10–18	20–25	Females and/or males	<14 †	<24 †	30
Cod (*Gadus morhua*)	August–November	10–18	30–45	Females and/or males	<78 *	<151 *	74
Four spotted megrim (*Lepidorhombus boscii*)	September–October	11.7–12.7	18–22	Females and/or males	<12 *	<13 *	317
Dragonet (*Callionymus lyra*)	September–October	12.0–12.8	15–22	Females and/or males	<144 *	<202 *	159
Red mullet (*Mullus barbatus*)	April	13.3–15.3	12–18	Males	<85 *	<208 *	40
Plaice (*Pleuronectes platessa*)	January	18.5–22.5	7–10	Males	<71 †	<9.49 †	116
Haddock (*Melanogrammus aeglefinus*)	August	5–10	33–55	Females and/or males	<72 †/<215 *	<162 †/<421 *	20/23
Saithe (*Pollachius virens*)	September	5–10	40–100	Females and/or males	<57 †	<142 †	21
Herring (*Clupea harengus*)	November	5–10	22–33	Females and/or males	<10 †	<23 †	24

Table 4.3. Dose–response, background response, and sensitivity in experimental studies with gadoid fish

SPECIES	SUBSTANCE(S)	LOWEST–HIGHEST CONCENTRATIONS	EXPOSURE TIME	BASELINE/CONTROL (LEVEL/ACTIVITY)	INDUCTION (FOLD)	REFERENCE
Polar cod (*Boreogadus saida*) juvenile	Crude oil (Oseberg C)	~200 mg kg^{-1} (i.p. inj.)	10 and 21 d post inj.	~30 pmol min^{-1} mg^{-1}	~8 and ~2.5 (245 and 80 pmol min^{-1} mg^{-1})	George *et al.* (1995a)
Polar cod (*Boreogadus saida*) male	Crude oil (Oseberg C)	~200 mg kg^{-1} (oral)	21 d post exposure	28 pmol min^{-1} mg^{-1} ±6 (n=12)	~5 (132 ± 14 pmol min^{-1} mg^{-1})	George *et al.* (1995a)
Polar cod (*Boreogadus saida*) female	Crude oil (Oseberg C)	~200 mg kg^{-1} (oral)	21 d post exposure	8 pmol min^{-1} mg^{-1} ±2 (n=14)	~5 (42 ± 6 pmol min^{-1} mg^{-1})	George *et al.* (1995a)
Polar cod (*Boreogadus saida*) juvenile	β-Naphthoflavone	50 mg kg^{-1} (i.p. inj.)	21 d post inj.	~30 pmol min^{-1} mg^{-1}	~12.5 (380 pmol min^{-1} mg^{-1})	George *et al.* (1995a)
Cod (*Gadus morhua*) juvenile	2,3,7,8-TCDD	0.008 mg kg^{-1} oral dose twice, d 0 and d 4	9 and 17 d post exposure	55.4 (d 9) and 91.4 (d 17) pmol min^{-1} mg^{-1}	~4 and ~3 (230 and 277 pmol min^{-1} mg^{-1})	Hektoen *et al.* (1994)
Cod (*Gadus morhua*) juvenile	PCB105	10 mg kg^{-1} oral dose twice, d 0 and d 4	measure at d 9 and d 17	55.4 (d 9) and 91.4 (d 17) pmol min^{-1} mg^{-1}	1.5 and 1.2 pmol min^{-1} mg^{-1}	Bernhoft *et al.* (1994)
Cod (*Gadus morhua*) juvenile	β-Naphthoflavone	100 mg kg^{-1} (i.p. inj. at d 0 and d 4)	measure at d 7	84 pmol min^{-1} mg^{-1} ±8 (n =5)	~13 (1 074 ± 340 pmol min^{-1} mg^{-1})	Goksøyr *et al.* (1987)
Cod (*Gadus morhua*)	β-Naphthoflavone	100 mg kg^{-1} (2 i.p. inj.)	measure 3–4 d after last injection	40 pmol min^{-1} mg^{-1}	~72 (2 870 pmol min^{-1} mg^{-1})	Goksøyr *et al.* (1991b)
Cod (*Gadus morhua*) juvenile	Crude oil (North Sea)	0.06–1 ppm	30 d	~2 pmol min^{-1} mg^{-1}	~2–5.5 (~ 4–11 pmol min^{-1} mg^{-1})	Aas *et al.* (2000a)

†Subfraction S9. *Microsomes subfraction.

Table 4.4. Dose–response, background response, and sensitivity in field studies with gadoid fish

SPECIES	SUBSTANCE(S)	LOWEST–HIGHEST CONCENTRATIONS	EXPOSURE TIME	BASELINE/CONTROL (LEVEL/ACTIVITY)	INDUCTION (FOLD)	REFERENCE
Rockling (*Ciliata mustella*)	Crude oil (Gullfaks; MV "Braer" spill, Shetland)	85 000 tons spill 129 ± 38 ng g^{-1} dry wt. of PAHs (selected 2- and 3-ring) detected in muscle	3 months after spill	~160 ± 50 pmol min^{-1} mg^{-1}	~9 (1 480 pmol min^{-1} mg^{-1})	George *et al.* (1995b)
Roundnose grenadier (*Coryphaenoides rupestris*)	i.a. PAHs and PCBs			260 ± 20 (male) and ~170 (female) pmol min^{-1} mg^{-1}	~2 (530 ± 70 (male) and ~350 (female) pmol min^{-1} mg^{-1})	Lindesjoo *et al.* (1996)
Hake (*Urophycis* spp.)	Pollution (PAH) from oil platforms (Gulf of Mexico) <100 m from platforms			10.9 ± 6.4 and 11.7 ± 10.5 pmol min^{-1} mg^{-1} (>3 000 m from platforms)	<1 (10.6 ± 3.8 and 10.5 ± 7.1 pmol min^{-1} mg^{-1})	McDonald *et al.* (1996)

5 Background document: fish vitellogenin (Vtg) as a biomarker of exposure to xenoestrogens

Craig D. Robinson and Alexander P. Scott

5.1 Executive summary

5.1.1 The need for determining Vtg

Within the OSPAR Convention, the Joint Assessment and Monitoring Programme (JAMP) requires contracting parties to monitor the quality of the marine environment and the effects of activities or natural and anthropogenic inputs to it. The Hazardous Substances Strategy requires monitoring of certain priority chemicals and a "research effort on endocrine disruptors leading to the development of testing and assessment tools for identifying substances of concern and their occurrence and distribution and effect in the marine environment". The identification of potential endocrine disruptors and the quantification of their environmental concentrations is potentially a complicated and expensive process, involving tiered toxicological screening and testing and the development of sensitive chemical analysis techniques. An alternative approach is to assess whether or not effects on hormonal systems are occurring in the marine environment, and then address the question as to the identity of the causative compounds.

At the 2005 meeting of the OSPAR Working Group on Concentrations, Trends and Effects of Substances in the Marine Environment (SIME), the UK recommended this approach for assessing the occurrence, distribution, and effect of compounds that act as xenoestrogens (SIME, 2005). Xenoestrogens are exogenous compounds that act upon exposed organisms to produce responses usually associated with the action of natural oestrogen hormones. The recommended method is the measurement of the egg-yolk protein vitellogenin (Vtg) in the blood plasma of male fish. This protein is naturally produced by female fish in response to endogenous oestrogens and is incorporated into the developing eggs as yolk. Male fish exposed to oestrogens will also produce Vtg in the liver, but with no ovaries to sequester the protein, it accumulates in the blood from extremely low basal concentrations (<10 ng ml^{-1}) and can reach very high concentrations (>50 × 10^6 ng ml^{-1}). This range of response helps to make it a very sensitive, as well as a highly specific, marker of oestrogenic exposure.

5.1.2 Field study design

The recommendations of the existing JAMP Guidelines on Contaminant Monitoring in Biota and on Contaminant-specific Biological Effects Monitoring also apply, with minor modification, to designing a sampling strategy to assess xenoestrogen contamination. The existing guidelines recommend using cod (*Gadus morhua*) and dab (*Limanda limanda*) in monitoring surveys, with flounder (*Platichthys flesus*) specified as a suitable substitute. The guidelines suggest collecting at least 12 fish of one sex of a constant size range, that sampling is undertaken outside the breeding season, and that it always takes place at the same time of year. To allow an assessment of oestrogenic endocrine disruptors, cod (offshore) and flounder (estuaries) are the recommended species. Dab may potentially also be used as the offshore species. Additional requirements needed to allow assessment of xenoestrogen contamination and effects are that male fish should be used and that any surveys take place in January/February, prior to the offshore migration of flounder.

5.1.3 The recommended method of determining Vtg

It is recommended that the Vtg in male fish plasma be determined by enzyme-linked immunosorbent assay (ELISA). There are many different types of ELISA assay, each with advantages and disadvantages; therefore, a standard operating protocol should be used for each species. The Vtg ELISA should be sensitive enough to quantify basal Vtg concentrations (i.e. have detection limits <10 ng ml^{-1}). It is also strongly recommended that all laboratories use a common supplier of Vtg antibody and antigen for each species; this is to remove analytical differences resulting from the purification of slightly different mixes of the different Vtg proteins produced by each fish species. For cod, an ELISA using lipovitellin is recommended because of the instability of Vtg from this species.

5.1.4 Supporting parameters

As with the other biological effects techniques within the Coordinated Environmental Monitoring Programme (CEMP), data should be collected for a range of supporting parameters and cofactors. These include the location (latitude and longitude) and date of collection, water temperature at collection site, unique fish identifier, gender, notes of any grossly visible anomalies and lesions, fish weight and length, gonadosomatic index, and the age of the fish. Sex should be confirmed, and the occurrence and severity of intersex assessed by histological examination of a gonad. Bile samples can be used in bioassay-directed fractionation and analysis in order to identify any oestrogenic metabolites. If mRNA transcripts are to be quantitated, a liver sample should be preserved in liquid nitrogen. To allow integrated monitoring of contaminants and effects, further samples of bile, liver, and muscle would be required for chemical analysis and for the determination of other biological effects measurements, such as EROD and metallothionein.

5.1.5 Applicability across the OSPAR maritime region

Cod, dab, and flounder can be caught across the OSPAR area. A common source of Vtg antigen and antibody (Lv for cod) for use in a defined standard protocol is required to allow between-laboratory data comparison. The development of the antibody, antigen, and protocol should be undertaken by an experienced laboratory. The establishment and validation of a standard ELISA protocol then ought to be straightforward for most laboratories.

5.1.6 Quality assurance

In order to have confidence in the determination of Vtg in male fish, suitable analytical control and proficiency must be demonstrated. This can be achieved by charting the results from the repeated analysis of internal laboratory reference materials and by the participation in the BEQUALM laboratory proficiency scheme. BEQUALM has undertaken one ring-test for cod Vtg assays, with the five participants producing comparable results. However, if Vtg is to be included as a CEMP determinant, flounder (and dab) Vtg must be added to the BEQUALM scheme and more laboratories encouraged to participate.

5.1.7 Assessment criteria

Environmental assessment criteria (EAC) cannot currently be set because of a lack of scientific agreement as to whether or not Vtg is a marker of exposure or effect, and because of an absence of data correlating Vtg concentrations with higher level effects in OSPAR sentinel fish species. Sufficient UK data are available to be able to propose a provisional background concentration of 0.13 μg ml^{-1} for Vtg in male flounder. Fewer data are available for cod, and a more tentative background concentration of 0.23 μg ml^{-1} is proposed, based upon a single study. Data for dab are not yet available, although one study is nearing completion. These figures should be reviewed once data conforming to the recommended sampling and quality assurance (QA) regime are available for the wider OSPAR area.

5.2 Introduction

Under the Convention for the Protection of the Marine Environment of the Northeast Atlantic (the "OSPAR Convention"), which came into force in 1998, the 16 contracting parties have agreed six strategies (OSPAR, 2003) by which they will "take all possible steps to prevent and eliminate pollution and to take the necessary measures to protect the maritime area against adverse effects of human activities so as to safeguard human health and to conserve marine ecosystems and, when practicable, restore marine areas which have been adversely affected". This document addresses the use of Vtg assays as measures of the biological effects of endocrine-disrupting chemicals, specifically xenoestrogens, in OSPAR-wide monitoring. As such, it is relevant to issues raised under the OSPAR JAMP and Hazardous Substances Strategy (HSS).

The JAMP assessments include evaluating the effectiveness of measures taken and planned for the protection of the marine environment, through the repeated measurement of

- the quality of the marine environment and each of its compartments;
- activities or natural and anthropogenic inputs that may affect the quality of the marine environment; and
- the effects of such activities and inputs.

Participation in a CEMP ensures that environmental monitoring by the contracting parties addresses the aims of the JAMP.

The OSPAR HSS aims to achieve concentrations in the marine environment near background values for naturally occurring substances and close to zero for synthetic substances (OSPAR, 2003). Included within the HSS are lists of "Chemicals for Priority Action" and "Substances of Possible Concern". These include some compounds selected because of their effects on endocrine systems. Under the HSS, the OSPAR Commission will "collaborate with various international forums with a view to optimizing international research effort on endocrine disruptors leading to the development of testing and assessment tools for identifying substances of concern and their occurrence and distribution and effect in the marine environment".

Testing compounds for hormonal effects and then deciding which should be monitored in the environment is very costly, as it would require thousands of compounds to be screened and tested for their hormonal effects, and then the development of analytical techniques to determine the environmental concentrations of selected compounds. An alternative approach is to determine whether or not biological effects are occurring, and then to identify the compounds responsible; this involves less toxicological testing and has the added benefits of indicating contaminant bioavailability and of measuring effects of exposure to chemical mixtures. The use of biological effects monitoring to investigate whether or not endocrine disruptor (ED) compounds are present in the environment is also consistent with the European Union Water Framework Directive (EU, 2000).

Monitoring of priority chemicals is required under the CEMP. Several priority chemicals or groups of chemicals are endocrine disruptors. The List of Substances of Possible Concern includes synthetic hormones (e.g. diethylstilbestrol and ethynyloestradiol), natural hormones (oestradiol and oestrone), and other compounds that have ED action (e.g. bisphenol A). Most of these compounds are xenoestrogens and cause biological effects through their interaction with the oestrogen receptor. The use of a biological effects measurement (Vtg) can allow an assessment of the occurrence, distribution, and effect of xenoestrogenic endocrine disruptors in the OSPAR maritime environment; the individual problem compound(s) could subsequently be identified and concentrations determined at targeted sites. Such an approach was recommended in a report from the UK to the March 2005 SIME meeting (SIME, 2005).

The 2005 SIME meeting also produced terms of reference (OSPAR, 2005) for a review of the biological effects component of the CEMP, requiring the development of background documents for biological effects monitoring techniques currently included in the CEMP. The meeting also asked the UK to elaborate on how to apply techniques for measuring Vtg in OSPAR monitoring and invited parties with experience using Vtg to inform the SIME 2006 meeting of their experiences. Several contracting parties indicated that they already undertake monitoring of xenoestrogens, or are considering so doing, by the determination of the egg-yolk protein Vtg in male fish, and there is existing recognition within ICES of the usefulness of Vtg measurements in biological effects monitoring (Scott and Hylland, 2002; ICES, 2005a). Building upon experience from its Endocrine Disruptors in the Marine Environment (EDMAR) programme, the UK volunteered to develop an OSPAR background document on the use of Vtg as a tool for assessing xenoestrogens in the marine environment.

5.3 Background scientific information

There are a number of publications reviewing the hormonal control of vitellogenesis and the role of Vtg in fish that have been used in preparing this report. The reader is referred to the following for the primary sources of information: Specker and Sullivan (1994), LaFleur (1999), Jalabert *et al*. (2000), Arukwe and Goksøyr (2003), Rotchell and Ostrander (2003), Hiramatsu *et al*. (2005), and Jalabert (2005). Additional sources of information are cited individually in the text.

5.3.1 Vitellogenin—definition and properties

Vitellogenin (Vtg) is a large, calcium-rich, phospholipoglycoprotein that is produced by the liver of female oviparous vertebrates in response to increases in circulating oestrogens. It is transported in the bloodstream to the ovary, where it is sequestered by developing oocytes and processed to form egg yolk. The main elements involved in the production and utilization of Vtg during oocyte maturation are shown in Figure 5.1 and described below. Concentrations of Vtg in the blood plasma of vitellogenic female fish can be six orders of magnitude higher than in immature or male fish. The production and utilization of the optimal quality and quantity of Vtg is thus critical to the reproductive success of most fish species.

As well as being the major source of amino acids and lipids for the developing embryo and a nutrient reserve for hatched larvae, Vtg also binds cations, such as

Ca, Mg, Zn, and Fe, and transports them into the oocyte for use during future embryogenesis. It binds steroid and thyroid hormones (Scott *et al.*, 1995; Cyr and Eales, 1996; Tagawa *et al.*, 2000) and may transport these into the egg to control embryo development.

In blood plasma, Vtg is transported as a dimer with a molecular mass of 300–600 kDa that is readily cleaved into the monomer protein (150–200 kDa). It consists of approximately 80 % protein and 20 % lipid (of which 70 % is phospholipids) and includes three main component yolk proteins, two lipid-rich (lipovitellin I, Lv-I; lipovitellin II, Lv-II), and one phosphorus-rich (phosvitin, Pv). Sequence homology indicates that Lv-I and Lv-II are more conserved between taxa than is the Pv domain.

When examined by polyacrylamide gel electrophoresis (PAGE), several smaller bands are also present, often owing to breakdown of Vtg that may occur during collection, purification, and/or storage. In some species, the breakdown is increased dramatically by the process of freeze-drying (Arukwe and Goksøyr, 2003). In some species there are two major bands between 150 and 200 kDa, indicating the presence of more than one type of Vtg. Species such as white perch (*Morone americana;* Hiramatsu *et al.*, 2002) and mosquito fish (*Gambusia affinis*; Sawaguchi *et al.*, 2005) possess three types of Vtg (denoted VtgA, VtgB, and VtgC), whereas others such as Japanese goby (*Tridentiger trigonocephalus*) have two types (Ohkubo *et al.*, 2003). VtgC lacks phosvitin.

5.3.2 Reasons for natural occurrence

Vtg belongs to an ancient gene family found in many invertebrates as well as all oviparous vertebrates. Following changes in environmental stimuli (Figure 5.1), such as photoperiod, temperature, or food availability, the hypothalamus secretes gonadotrophin-releasing hormone (GnRH) to stimulate the pituitary to synthesize and release gonadotrophin hormones (GtH I and GtH II). These cause the immature ovary to release the hormone oestradiol (E2) into the bloodstream. Oviparous females produce Vtg in response to the rise in circulating E2 titres. Within liver cells, E2 binds to the nuclear oestrogen receptor (ER), which dimerizes and binds to the oestrogen-responsive element (ERE) of the promoter region of E2-inducible genes, including Vtg and the ER itself. The binding of the ER to the ERE thus results in increased mRNA transcription and consequent production of E2-inducible proteins. Hepatically produced Vtg is transported in the bloodstream to the ovary and is sequestered by the developing oocytes. The incorporation of large amounts of Vtg into the oocytes results in the characteristic increase in the size of the ovaries during sexual maturation.

During oocyte maturation, proteolysis of Lv derived from VtgA is used to generate free amino acids that, by increasing the osmotic pressure within the oocytes, draw in water. Selective proteolysis of yolk proteins gives rise to distinct differences in the free amino acid profiles of pelagic and demersal eggs (Finn *et al.*, 2002) and accounts for their differing buoyancy, as proteolysis is especially important in fish with pelagic eggs (Reith *et al.*, 2001; Matsubara *et al.*, 2003). The Lv derived from VtgB is mainly laid down in the form of yolk within membrane-bound platelets, and there is evidence that the developing embryo utilizes Lv-derived lipid, whereas the protein component is reserved for larval use (Hartling and Kunkel, 1999).

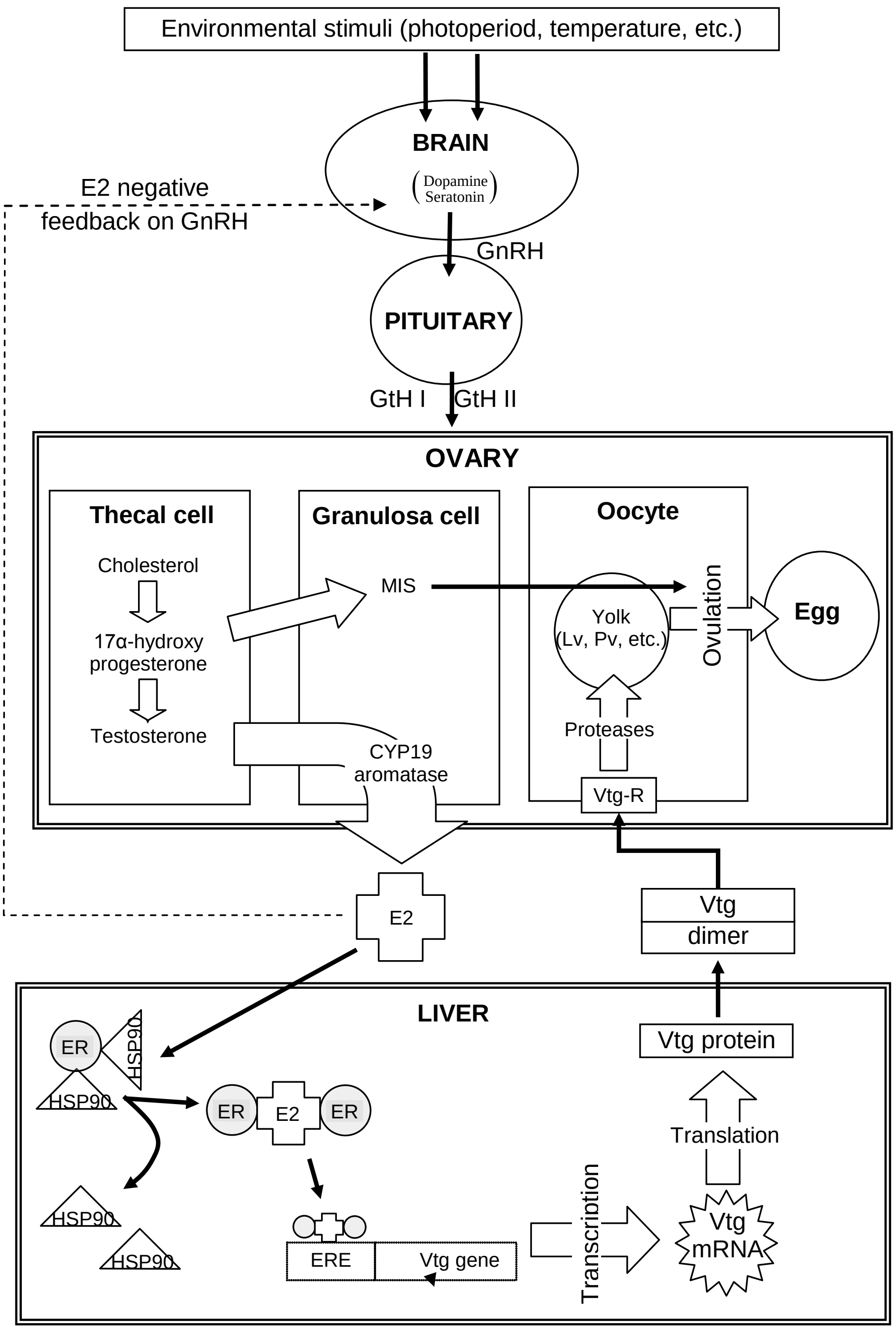

Figure 5.1. Diagram of the brain–pituitary–gonad–liver axis in female teleosts, showing the main elements involved in production and utilization of vitellogenin (Vtg) during oocyte maturation. E2, 17β-oestradiol; ER, oestrogen receptor; ERE, oestrogen-responsive element; GnRH, gonadotrophin-releasing hormone; GtH, gonadotrophin hormone; HSP, heat-shock proteins; Lv, lipovitellin; MIS, maturation-inducing steroid; Pv, phosvitin; Vtg, vitellogenin; Vtg-R, vitellogenin receptor.

5.3.3 Vtg response of fish to contaminant exposure

Hepatic Vtg production in female fish is controlled by 17β-oestradiol (E2). However, Vtg genes are also present in male fish, and the artificial administration of E2 to immature or male fish results in dose-responsive increases in Vtg mRNA expression and plasma Vtg titres. In most species Vtg concentrations in plasma can rise a million-fold in response to oestrogen stimulation. No other recorded biological effect has such a high range of response. Even very low doses of oestrogen can result in large increases in plasma Vtg, making the determination of Vtg in male fish an extremely sensitive marker of oestrogen exposure (Sumpter and Jobling, 1995).

Many natural and artificial compounds are able to act as oestrogens. The degree to which an oestrogenic compound is able to induce Vtg is governed by its ability to bind to the hepatic oestrogen receptor alpha (ERα). Many compounds (e.g. alkylphenols, many halogenated organic compounds, certain pesticides, some phthalate plasticizers, paraben preservatives, and phytosterols) are weakly oestrogenic, whereas a few pharmaceutical compounds (e.g. EE2, DES) may be more potent than E2 itself. Some organochlorine compounds, including *o,p'*-DDT, methoxychlor, and certain PCB congeners, are themselves very weak ER agonists, but their hydrolysed metabolites are much stronger ER agonists (Bulger *et al.*, 1978; Korach *et al.*, 1988; Soontornchat *et al.*, 1994), although, as metabolites, they can be expected to be rapidly eliminated from the body.

ER agonists have the same mechanism of action; thus, exposure to mixtures of exogenous oestrogen mimics results in increased Vtg response. The effects of exposure to multiple oestrogenic compounds are additive, based upon the relative concentrations and potencies of the individual components of the mixture (Payne *et al.*, 2000; Thorpe *et al.*, 2003; Brian *et al.*, 2005). Consequently, even when the individual compounds are each below the threshold concentration that causes Vtg induction, the overall potency of the mixture may be sufficient to induce Vtg. This allows the potencies of oestrogenic mixtures to be determined (*in vitro* or *in vivo*) in terms of E2-equivalents (e.g. ng E2-equivalents l^{-1}) in a manner analogous to the determination of toxic equivalents for planar organic compounds. Importantly, it also allows an assessment of whether or not fish are being exposed to xenoestrogens in the environment, even when the concentrations of individual compounds may be very low and difficult to detect through chemical analysis. This is one of the key reasons for recommending the determination of Vtg in male fish as a method allowing the monitoring and assessment of the occurrence and effects of oestrogenic EDs in the marine environment.

Coexposure with certain other compounds can result, however, in perturbations to this model. For example, some compounds block the binding of E2 to the ER and thus are anti-oestrogenic. Others (e.g. some planar organic compounds, certain metals) have indirect anti-oestrogenic effects. Many polycyclic aromatic hydrocarbons (PAHs) and other planar organic compounds that interact with the aryl hydrocarbon receptor (AhR) to cause induction of the cytochrome P450 1A (CYP1A) detoxification enzyme system are also believed to be indirectly anti-oestrogenic. Female exposure to these compounds can lead to increased E2 metabolism and reduced plasma E2 titres, thus inhibiting Vtg production, impairing oocyte development, and reducing reproductive output (reviewed in Nicolas, 1999). The inhibition of oestrogenic effects by AhR agonists may also be the result of a reduction in ERα expression (Bermanian *et al.*, 2004). The effects of simultaneous exposure to ER agonist(s) and AhR agonist(s) on Vtg expression in fish are complex and not well understood. Responses appear to depend upon the relative ratios of ER agonist to AhR agonist, the sex and maturational state of the fish, and the sequence of exposures (Anderson *et al.*, 1996; Arukwe and Goksøyr, 2003). Despite these complications, male flounder from UK estuaries contaminated by AhR inducers and ER agonists demonstrate both elevated plasma Vtg (Kirby *et al.*, 2004a) and induced CYP1A enzyme activity (Kirby *et al.*, 2004b). Thus, Vtg can be used as a marker of oestrogenic exposure in environments that are also contaminated with AhR inducers. Recent unpublished laboratory studies by Kirby *et al.* have additionally demonstrated that AhR inducers had no influence on the ability of E2 to induce Vtg in immature flounders. However, E2 was very effective at inhibiting the ability of AhRs to induce CYP1A.

5.3.4 Existing evidence of the effect of oestrogenic EDs on marine fish

There is now a large body of evidence of the existence of oestrogenic endocrine disruption in the marine environment. Male flounder (*Platichthys flesus*) caught in industrialized estuaries of the UK and the Netherlands have been found with elevated concentrations of Vtg in their plasma (Lye *et al*., 1997, 1998; Matthiessen *et al*., 1998a; Allen *et al*., 1999a,b; Vethaak *et al*., 2002, 2005; Kirby *et al*., 2004a; Kleinkauf *et al*., 2004). The range of Vtg concentrations that has been measured is extremely wide (from < 10 ng ml^{-1} to > 50 × 10^6 ng ml^{-1}). Some male flounder with elevated Vtg concentrations have also been caught in the open sea (Allen *et al*., 1999a), but these were hypothesized to be fish that had recently emigrated from a contaminated estuary. In estuarine and coastal areas of the USA (Mills *et al*., 2003; Roy *et al*., 2003) and Japan (Hashimoto *et al*., 2000; Hara *et al*., 2001; Ohkubo *et al*., 2003), many fish have also been found with high concentrations of Vtg in their plasma. There is evidence that cod from some inshore areas of Norway have elevated Vtg (Scott *et al*., 2006a).

In the open seas, oestrogenic effects have been observed in swordfish (*Xiphias gladius*) from the Mediterranean (Fossi *et al*., 2001, 2002, 2004; Desantis *et al*., 2005) and off the coast of South Africa (Desantis *et al*., 2005), but not in the Pacific Ocean (Desantis *et al*., 2005). Similarly, many male tuna (*Thunnus thynnus*) caught in the Mediterranean had Vtg in their plasma (Fossi *et al*., 2002), whereas tuna (*Thunnus obesus*) caught in the Pacific Ocean (Hashimoto *et al*., 2003) did not. Plasma Vtg concentrations in male cod from the Northeast Atlantic show a positive correlation with size, possibly the result of changes in diet as they grow or caused by long-term accumulation of contaminants (Scott *et al*., 2006a).

In addition to direct evidence of oestrogenic effects (i.e. Vtg protein or mRNA in males), there is also indirect evidence. The presence in males of intersex gonads (Lye *et al*., 1997; Allen *et al*., 1999a; Cho *et al*., 2003; Metrio *et al*., 2003) or feminized secondary sexual characteristics (Kirby *et al*., 2003) also suggests oestrogenic exposure. However, sexual differentiation in fish is very plastic. Other types of compounds (e.g. aromatase inhibitors, androgens, and antiandrogens), or even temperature change, are able to alter fish gender (Baroiller *et al*., 1999; Al-Ablani and Phelps, 2002). In contrast, only oestrogenic compounds are so far known to be involved in the induction of Vtg (Sumpter and Jobling, 1995). Field studies of flounder in the UK have indicated a poor association between Vtg induction and male intersex (Allen *et al*., 1999b). For example, at a site with a prolonged history of high Vtg induction (the Tees estuary in the UK), hermaphrodites have yet to be recorded (Kirby *et al*., 2004a).

5.4 Methodology

5.4.1 Design of field surveys

The existing JAMP Guidelines for Monitoring Contaminants in Biota, or Contaminant-specific Biological Effects (OSPAR agreements 1999–2002 and 2003–2010) can be readily adapted for use in the assessment of oestrogenic exposure through the determination of Vtg in male fish. The similarity between the Guidelines and the benefits of integrating chemical analysis with biological effects determinations has already been noted. For this reason, the 2005 (ICES, 2005b) and the 2006 (ICES, 2006a) meetings of the Joint ICES/OSPAR Workshop on Integrated Monitoring (WIKIMON) produced draft JAMP Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects ("The MERGED Guidelines"). Further advice on the use of Vtg in biological effects monitoring, including field-survey design, is available in the form of an *ICES Techniques in Marine Science* (*TIMES*) paper (Scott and Hylland, 2002).

The existing JAMP Guidelines recommend using cod (*Gadus morhua*) and dab (*Limanda limanda*) in monitoring surveys, with flounder (*Platichthys flesus*) specified as a suitable substitute for dab. The guidelines suggest collecting at least 12 fish of one sex and of a constant size range; that sampling is undertaken outside the breeding season, and that it always takes place at the same time of year. These recommendations (for reasons outlined below) would also apply when designing a sampling strategy to assess xenoestrogen contamination. Additional requirements are for male fish to be used and for any survey to be conducted in late winter / early spring.

There are sufficient data from the UK and elsewhere to demonstrate that male flounder are suitable for the assessment of xenoestrogenic contamination of estuarine environments (e.g. Allen *et al.*, 1999a,b; Vethaak *et al.*, 2002, 2005; Kirby *et al.*, 2004a; Kleinkauf *et al.*, 2004). Preliminary data from caged fish in the BECPELAG project (Scott *et al.*, 2006b) and a further survey of wild fish (Scott *et al.*, 2006a) suggest that male cod may be suitable for use in offshore areas. The UK has recently developed an ELISA for dab, and initial findings suggest that this species may also be suitable as a sentinel in monitoring oestrogenic contamination, although the development of a more sensitive ELISA will be necessary since Vtg concentrations are, on average, lower than those found in cod (A. P. Scott, pers. comm.).

UK data for flounder indicate a marked seasonality in male plasma Vtg concentrations, with concentrations being highest in February/March and lowest in June/July (Kirby *et al.*, 2004a; Kleinkauf *et al.*, 2004). This reflects migration of the fish to and from the estuary and indicates that annual surveys of flounder Vtg must take place just before the fish are expected to migrate offshore to breed (i.e. in late winter / early spring).

There is evidence that plasma Vtg concentrations are correlated with fish size for species caught away from point sources, for example in dab (A. P. Scott, pers. comm.), cod (Scott *et al.*, 2006a), tuna, and other species from high trophic levels (Barucca *et al.*, 2006). To reduce biological variability, it is recommended that only fish from a narrow size range be analysed for Vtg. To assess recent xenoestrogen contamination, smaller cod (30–45 cm) should be sampled, as recommended in the existing JAMP Guidelines.

Spermiating male plaice can show slight Vtg induction (Scott *et al.*, 1999) that is thought to be related to either endogenous production of E2 (Wingfield and Grimm, 1977) and/or to androgen–ER interactions (Kim *et al.*, 2003); for this reason, Vtg surveys should avoid the breeding season. At contaminated sites there are often large (several orders of magnitude) interindividual variations in Vtg concentrations because of some fish responding and others not (e.g. Allen *et al.*, 1999a). This causes difficulties in statistical analysis because of the large heterogeneity of variances between sites, which could be the result of interindividual differences in genotype, size, migration, or prey selection. This variability and lack of response in some fish emphasizes the requirement to reduce the biological variability as much as possible through careful design of the survey and sampling strategies and the need for the analytical technique to be able to determine basal Vtg concentrations. Many of the requirements of a Vtg monitoring survey are the same as those included in the JAMP Guidelines for other CEMP determinants, as can be seen in Table 5.1.

5.4.2 Sample collection

As noted above, Vtg is present in plasma as a dimer and is naturally cleaved in the oocytes to form individual yolk proteins. Because of this, Vtg is relatively unstable, and blood samples must be collected and handled in a rigorously standardized manner to avoid damage to the protein. Blood should be collected into heparinized tubes, stored immediately on ice, and centrifuged within 30 min. Protease inhibitors must be added to the plasma, which should be divided into two subsamples, snap-frozen in liquid nitrogen, and the two subsamples stored in separate cryogenic freezers or liquid nitrogen cryostores. Samples must not be allowed to thaw prior to assay.

5.4.3 Determination of Vtg

The expression of Vtg genes can be measured either as mRNA transcripts or as protein, and either endpoint can be estimated semi-quantitatively, or accurately quantified. The induction of Vtg mRNA transcription is measurable within a few hours of exposure, and decays rapidly ($t_{½}$ = 3–4 d) once the exposure ceases. In comparison, plasma Vtg titres take several days to reach measurable concentrations and decay with a half-life of days to weeks (Craft *et al*., 2004), such that males may take several months to clear Vtg from their bloodstream. A low plasma Vtg concentration may thus indicate a recent small exposure or a larger exposure some time in the past.

5.4.3.1 Determination of hepatic Vtg mRNA

The induction of hepatic Vtg mRNA can be measured either semi-quantitatively through reverse transcription polymerase chain reaction (RT-PCR) or quantitatively with real-time PCR (Q-PCR). In both methods, a cDNA probe is hybridized with mRNA from samples and standards and then amplified through PCR. Measurement of mRNA requires specialized equipment and access to Vtg cDNA probes for each species; although internal quality control methods may be used, there is currently no external QA scheme available. Therefore, Vtg mRNA cannot be currently recommended for use in OSPAR integrated monitoring.

5.4.3.2 Determination of plasma Vtg concentrations

Vtg protein titres in blood plasma can be indirectly estimated by determining total protein, Ca, or P concentrations. However, these are not specific measures of Vtg, are not sufficiently sensitive to quantify basal Vtg concentrations, and are thus not suitable for use in environmental monitoring of oestrogenic exposure.

Recent advances in proteomic and chemical analysis have led to instrumental means of assessing Vtg. However, these techniques involve specialized instrumentation (e.g. liquid chromatography combined with tandem mass spectrometry) that is not readily available to most monitoring laboratories; therefore, immunoassays are a much more commonly used technique for Vtg determination.

Since Vtg is highly antigenic, quantitative methods of determination based upon immunoassay are relatively straightforward to develop, and many are described in the scientific literature (e.g. Mañanós *et al*., 1994; Allen *et al*., 1999a; Fujiwara *et al*., 2005). Specker and Anderson (1994) and Hiramatsu *et al*. (2005) detail how to develop each of the different types of ELISA procedures that are available. Some types are more sensitive assays than others. ICES has published guidelines on the use of Vtg determinations in biological effects monitoring programmes (Scott and Hylland, 2002). Immunoassays are very sensitive and may have a high specificity. The specificity is ultimately controlled by the purity of the Vtg used for radiolabelling (in radioimmunoassays), for plate-coating (for competitive ELISAs), or for raising the antibodies (for sandwich ELISAs).

To increase the stability of Vtg it is usual to add protease inhibitors (e.g. aprotinin or PMSF) and to keep it cold during all stages of handling. Apparent success in the purification of Vtg (e.g. as a single band on an electrophoresis gel) may be the result of these precautionary measures, but can also be the result of some species having more robust Vtg than others (Silversand *et al.*, 1993; Hennies *et al.*, 2003). Cod Vtg appears to be particularly difficult to prepare in an intact form (Silversand *et al.*, 1993; Arukwe and Goksøyr, 2003), leading to the use of Lv as the standard antigen in immunoassays (Meier *et al.*, 2002; Scott *et al.*, 2006a). Vtg from some other species (e.g. flounder) have proven to be relatively robust proteins during the processes of purification and storage and thus much easier to develop into immunoassays for monitoring purposes (e.g. Matthiessen *et al.*, 1998b). Validated Vtg radioimmunoassay (RIA) and ELISA assays tend to have inter- and intra-assay variations (5–20 %) that are insignificant compared with the concentrations in exposed fish (e.g. Matthiessen *et al.*, 1998b; Parks *et al.*, 1999; Mosconi *et al.*, 2002; Eidem *et al.*, 2005).

To date, it has been unclear in most fish Vtg studies whether or not the assay has been measuring VtgA, VtgB, VtgC (see above), or a mixture of all three. In only three species (tilapia, Japanese goby (*Tridentiger trigonocephalus*), and medaka (*Oryzias latipes*)) have assays been developed for the different forms (Takemura and Kim, 2001; Ohkubo *et al.*, 2003; Fujiwara *et al.*, 2005). In the tilapia and Japanese goby, VtgA responds to lower doses of E2 and reaches higher levels than VtgC. Similarly, in medaka, VtgA has been demonstrated to reach tenfold higher levels in the plasma in response to E2 than VtgB. These differences mean that if two laboratories use antibodies and antigens that have slightly different VtgA : VtgB : VtgC ratios, they could produce different results for a common sample. It is difficult and expensive to purify a specific Vtg; therefore, it is recommended to use a common source of Vtg antibody and antigen in Vtg surveys, rather than specify that the assay to be used should be directed against, for example, VtgA. In this case, the precise mix of Vtg proteins present will not matter, as it will be the same in all testing laboratories. The detection limit and inter-/intra-assay variation should be quantified and interlaboratory comparisons made through ring-tests (e.g. external QA schemes such as BEQUALM, see below).

Analytical ELISA kits (including antibodies, standards, and reagents) for the determination of Vtg are available for several fish species, including salmon, medaka, zebrafish, fathead minnow, carp, rainbow trout, and cod (Biosense, Norway; Amersham Biosciences, UK) and are straightforward for a suitably equipped laboratory to employ. The commercially available cod assay attempts to measure intact Vtg. However, this is particularly unstable in cod; for this reason, an ELISA based upon Lv is recommended. Unfortunately, ELISA kits are not currently commercially available for flatfish species used in routine OSPAR monitoring programmes. If Vtg is added to the list of determinants required by the OSPAR CEMP, this may create sufficient demand for commercial suppliers to provide a flatfish Vtg ELISA. In addition, for the technical reasons given above, all of the participants in a monitoring programme should use a common source of Vtg antibody and antigen.

5.5 Supporting parameters

In order to aid interpretation of the data produced, the collection of data for a number of supporting parameters and cofactors is recommended. Each fish must be given a unique identifier and its sampling location (site name, site code, latitude, and longitude) recorded; any grossly visible anomalies and lesions should be noted. Biological variability will be reduced by sampling plasma from only male fish of a specified size range and at a specified time of year. However, the length, weight, and gonad weight of each individual fish should be recorded. Recording gonad weight allows the calculation of the gonadosomatic index (gonad weight as a percentage of body weight), an indication of the sexual maturity of the fish. A gonad should also be preserved to allow histological examination, both to confirm sex in the event of a high Vtg titre and in order to determine the incidence and severity of intersex (presence of oocytes in the testes). Systems to describe the severity of intersex have been developed for flounder (Bateman *et al.*, 2004) and roach (*Rutilus rutilus*; Bjerregaard *et al.*, 2006), and can be adapted for use with other species, such as dab (Stentiford and Feist, 2005). Otolith samples should also be taken in order to age each fish. Bile samples could be collected to allow bioassay-directed fractionation and identification of any oestrogenic metabolites present. If mRNA transcripts are to be quantitated, a liver sample should be preserved in liquid nitrogen. As part of an integrated ED monitoring programme, additional samples of liver and muscle would be required for chemical analysis.

Bioassay-directed fractionation and identification of oestrogenic metabolites involves chemical extraction and chromatographic fractionation of the extracts coupled to the use of *in vitro* assays, such as the yeast oestrogen screen (YES), to identify any oestrogenically active fractions of the extracts. The active compounds can be further separated and subsequently identified using mass spectrometry. Following the findings of male Vtg and intersex in male freshwater fish in UK rivers (e.g. Purdom *et al.*, 1994; Harries *et al.*, 1996, 1997), this technique was used to identify the major oestrogenic compounds in sewage treatment work effluents (Desbrow *et al.*, 1998). It can also be applied to sediment extracts (Thomas *et al.*, 2004) in order to identify oestrogenic compounds present in the environment, or to bile samples to identify bioavailable oestrogenic compounds.

5.6 Applicability across the OSPAR maritime area

5.6.1 Geographical considerations

The species recommended for use in assessing the occurrence and effects of xenoestrogenic endocrine disruptors (flounder and cod) are already recognized as being suitable for OSPAR-wide contaminant and biological effects monitoring. This is the result of their widespread distribution and life habits. Flounder are recommended as the sentinel species for estuarine environments, as they are known to occur throughout the OSPAR region and are responsive to oestrogenic exposure. However, they are not continually resident in estuaries because they migrate offshore to breed, and in order to sample following the maximum period of potential exposure, any survey should take place prior to the emigration. It is not known, however, whether all of the adult fish of a population migrate, nor is the exact timing of the emigration known for all populations. Cod are widely distributed within the OSPAR region and are the recommended species for monitoring xenoestrogen contamination in offshore areas. Dab may also be suitable for offshore monitoring.

5.6.2 Technical considerations

Validation of a recognized Vtg ELISA, for which the protocol, antigen, and antibodies are available, is relatively straightforward for a laboratory with ecotoxicological experience. The only equipment required is a centrifuge, 96-well plate reader, and cryogenic storage facilities. An automated 96-well plate washer is advantageous as it makes the procedure less laborious. The laboratory will need to demonstrate parallel dilution curves for plasma samples and standards, determine robust limits of detection and limits of quantification, and develop internal QA procedures using control charts to demonstrate statistical control of the assay. This may involve the analysis of a laboratory reference material on each 96-well plate, or the reanalysis of a sample from the previously analysed batch. To develop laboratory reference material, one or more fish are induced to produce Vtg by repeated weekly injection with oestradiol (e.g. three injections of 5 mg kg^{-1} E2, dissolved in a carrier such as corn oil or squalene). After a further week, blood is collected into heparinized tubes, protease inhibitors added, and the samples centrifuged to obtain plasma. The plasma samples are thoroughly mixed together and homogeneous aliquots frozen. Long-term storage requires an alarmed cryogenic freezer as a minimum; storage in liquid nitrogen is preferred because it is colder and less liable to failure. Each aliquot of laboratory reference material plasma is assayed once only.

Should a common source of antibody and antigen not be available, each laboratory will need to develop its own antibody and standard antigen. As already noted, this is not recommended as it reduces the reliability of simple comparison between results from different laboratories. However, many Vtg assays are described in the literature (e.g. Mañanós *et al.*, 1994; Allen *et al.*, 1999a; Fujiwara *et al.*, 2005). Briefly, the procedure requires purified Vtg to be obtained for the species of interest, the raising of an antibody to the Vtg, demonstration of its specificity for Vtg, and full validation of the assay (including external QA) as described above and detailed elsewhere (e.g. Specker and Anderson, 1994; Hiramatsu *et al.*, 2005). Purification of Vtg requires specialized chromatographic instrumentation and a degree of experience in its use, which may restrict the numbers of laboratories able to develop their own Vtg assay.

5.7 External quality assurance

There are currently no certified reference materials available for Vtg in male fish plasma, although an external QA scheme is available to allow interlaboratory comparisons. This developed from an EU-funded project (Biological Effects Quality Assurance in Monitoring Programmes—BEQUALM) that ran from 1998 to 2002 and has since become a self-funding QA scheme for biological effects monitoring, similar to the QUASIMEME scheme for chemical analysis. The determination of plasma Vtg concentrations is included in this scheme, with the lead laboratory being the Norwegian Institute for Water Research (NIVA). The first Vtg ring-test under the self-funding programme took place in 2004–2005, with five laboratories returning data to the NIVA. Each laboratory received five plasma samples from Atlantic cod that were pooled from E2-treated fish, control fish, or a combination of induced and control. One of the five laboratories returned semi-quantitative data. All five laboratories were able to distinguish the three samples with induced Vtg from the two with low Vtg. The quantitative data showed good agreement in the concentrations determined for each of the three induced samples, with greater interlaboratory variability for the two uninduced samples, in which the Vtg concentrations were close to the limit of quantification. The five laboratories produced comparable results, and all would have been able to detect the effects of xenoestrogens on Vtg in Atlantic cod (NIVA, 2005). In order for Vtg determinations to be compatible with JAMP principles, the BEQUALM scheme should be expanded to include other fish species that are used in CEMP monitoring (e.g. flounder, dab) and a larger number of laboratories should be encouraged to participate.

5.8 Assessment criteria

At the 2004 Joint ICES/OSPAR Workshop on the Evaluation and Update of Background Reference Concentrations (BRCs) and Ecotoxicological Assessment Criteria (Moffat *et al*., 2004), it was agreed to replace the use of background/reference concentrations (BRCs) and ecotoxicological assessment criteria with background concentrations (BCs) and environmental assessment criteria (EAC). BCs are concentrations typical of remote areas, or (for contaminant data) from within sediment cores at depths deposited prior to industrialization. The Workshop also proposed that testing of whether or not mean observed concentrations are near-background concentrations should be carried out using "background assessment concentrations". These are statistical tools defined in relation to the BCs on the basis of the variability within the monitoring dataset and which allow testing of whether or not observed mean concentrations can be considered to be near to background concentrations. EAC are the concentrations below which there should be no deleterious effects on the exposed individual, population, or community.

Applied to Vtg, these definitions imply that the induction of Vtg in male fish is either a marker of oestrogenic exposure (BC) or a marker of harmful effect (EAC). Male Vtg induction is recognized as an indicator of oestrogenic exposure, but there is currently no scientific consensus that it is also an indicator of harmful effect. In laboratory studies on some fish species, Vtg tends to be more sensitive to oestrogenic exposure than gross reproductive endpoints, such as fecundity and fertility (e.g. Kang *et al*., 2002; Brion *et al*., 2004; Tilton *et al*., 2005), whereas in other reports, these reproductive endpoints have been demonstrated to be more sensitive than Vtg (e.g. Gronen *et al*., 1999; Cheek *et al*., 2001). Few reproductive studies have used marine fish, and none have used OSPAR sentinel species.

Field studies linking Vtg with reproductive effects are very rare. However, work on English sole (*Parophrys vetulus*) (presented by Johnson *et al*., at the SETAC North America 26th Annual Meeting in Baltimore, MD, USA, November 2005) showed that, at sites where males had elevated plasma Vtg, females began vitellogenesis early and matured more slowly than fish from clean sites. These results would appear to confirm earlier studies on flounders (Janssen *et al*., 1997) which showed that female fish experimentally exposed to contaminated harbour sediment for three years showed premature vitellogenesis and elevated plasma Vtg levels (although males in this case did not have elevated Vtg levels).

5.8.1 Environmental assessment criteria

To set EAC, plasma Vtg above a certain concentration needs to be demonstrated to have a deleterious effect on individuals, populations, or communities. In individual fish, very high plasma Vtg concentrations are associated with kidney failure and poor Ca homeostasis (Herman and Kincaid, 1988), reduced reproductive success, and histologically abnormal gonads (intersex). The correlation between plasma Vtg concentration and intersex is weak, because the timing of exposure is critical. Intersex is believed to be an irreversible condition and can be consequent to male fish having been exposed to oestrogens at early life stages during gonadal differentiation, whereas Vtg production can be induced at any age, and concentrations in the plasma decrease following cessation of exposure. A fish may have been exposed during gonadogenesis and be intersex, but it may not show Vtg induction if the exposure ceased several weeks before sampling.

A number of studies have examined the relationship between Vtg and reproductive success. However, model aquarium species used for life cycle testing (e.g. Japanese medaka, zebrafish, fathead minnow) are small, and blood plasma samples are difficult to obtain from them. Consequently, there are few studies correlating absolute plasma Vtg concentrations with reproductive output. Those that do have tended to use fathead minnow (*Pimephales promelas*), which is larger than the other species (e.g. Kramer *et al.*, 1998; Harries *et al.*, 2000; Länge *et al.*, 2001). Reproductive endpoints were affected when male fathead minnow plasma Vtg concentrations exceeded approximately 100 μg l^{-1} (Harries *et al.*, 2000) or 10 μg l^{-1} (Länge *et al.*, 2001). However, the relative sensitivities of the Vtg and reproductive endpoints, and the strength of the correlation between Vtg and reproductive effects depend upon many factors. These include the particular compound(s) tested, the timing and duration of the exposure in relation to the age of the fish and its reproductive status, the time of sampling in relation to both the time of exposure and the reproductive status, and upon the species being investigated (e.g. Segner *et al.*, 2003; Brion *et al.*, 2004).

Because there are no data available linking plasma Vtg and reproduction in CEMP-recommended sentinel organisms, and because several of the factors listed above are variable in the marine environment, it is unlikely that it will be possible to set reliable EAC for plasma Vtg concentration.

5.8.2 Background concentrations

Because of the lack of agreement over whether or not Vtg is a marker of effect, it should be, in theory, easier to establish a BC for plasma Vtg concentrations than to set EAC. There are limited environmental and laboratory data available for plasma Vtg concentrations in flounder and cod (Tables 5.2 and 5.3), suggesting that setting a BC should be possible. Vtg data for dab have not yet been submitted for peer review, although a study has recently been completed on dab from offshore UK waters (A. P. Scott, pers. comm.). A variety of factors including interspecific, seasonal, and size differences in Vtg concentrations (Hiramatsu *et al.*, 2005), plus the presence of phytoestrogens in commercial diets often used in laboratory studies (Pelissero *et al.*, 1989; Matsumoto *et al.*, 2004) and a paucity of data in marine fish, currently make it difficult to set a firm BC for the entire OSPAR region. However, sufficient data exist from studies in the UK to set a provisional BC for Vtg in male flounder, although this figure should be reviewed once additional data are obtained from field surveys that fulfil the requirements noted above. There is one field experiment (BECPELAG) from which data can be used to suggest a provisional BC for cod.

UK flounder data demonstrate variability within sites owing to non-standardized sampling months, fish sizes, etc. Nevertheless, a provisional BC of 0.13 μg ml^{-1} can be set, based upon the 90th percentile of the entire male Vtg concentrations (range = <0.01–0.17 μg ml^{-1}, $n = 95$) in fish collected at a UK reference estuary (River Alde) between 1996 and 2001. In the BECPELAG study (Scott *et al.*, 2006b), Vtg concentrations in caged male cod from reference sites in the North Sea were determined to be in the range <0.01–1.35 μg ml^{-1} ($n = 69$). Based upon the 90th percentile of these data, a provisional BC of 0.22 μg ml^{-1} is proposed for cod.

5.9 Concluding comments

In order to assess the occurrence, distribution, bioavailability, and effects of oestrogenic endocrine-disrupting chemicals in the OSPAR region, it is recommended that the determination of the egg-yolk protein Vtg in the blood plasma of male fish be added to the list of contaminant-specific biological effects included in the OSPAR CEMP.

The recommended sentinel species are flounder (estuaries) and cod or dab (offshore), which should be sampled between January and March, depending on location.

In addition to plasma samples, gonad samples should be sampled and histologically examined to allow the presence and severity of intersex to be determined.

Other sampling requirements are covered by existing JAMP Guidelines.

The recommended analytical method is ELISA, using purified Vtg as standard for flounder and dab, and lipovitellin (Lv) for cod.

A common source of Vtg antibody and antigen should be made available to the different contracting parties for the flounder and dab assays, and a common source of Lv antibody and antigen for the cod assay.

The existing BEQUALM quality assurance scheme includes the determination of Vtg in cod, and should be expanded to included flounder and dab.

Existing data from the UK allow a provisional background concentration for Vtg in male flounder plasma to be set at 0.13 $\mu g\ ml^{-1}$. The limited Vtg data for cod suggest a provisional BC of 0.22 $\mu g\ ml^{-1}$ for this species. These values should be reviewed as more data becomes available. Background assessment concentrations should be developed and used as the criteria against which to assess whether or not oestrogenic exposure is occurring.

Data in the scientific literature indicate that the suggested provisional BC is exceeded in flounder from several UK estuaries and some areas of the southern North Sea. This indicates that biologically significant concentrations of oestrogenic endocrine disruptors are present in these areas.

In areas where male fish exhibit elevated Vtg, the identity of the compounds responsible should be established by bioassay-directed fractionation and mass spectrometry of bile and/or sediment extracts.

Table 5.1. Requirements for Vtg monitoring compared with existing JAMP recommendations

	Existing JAMP recommendation	Recommendation for xenoestrogen assessment	Comment
Species and size	Cod, 30–45 cm Flounder, 15–35 cm Dab, 20–25 cm	Cod, 30–45 cm Flounder, 15–35 cm	Cod are suitable for offshore areas, but Vtg is related to size of fish, so sampled animals must be of a proscribed size range Flounder are suitable for estuarine areas Dab may be suitable, but no data are currently available
Sex	Single, either	Male	Females have naturally high Vtg concentrations that are too variable for them to be useful in monitoring (anti-) oestrogens in the environment
Time of year to sample	Outside the breeding season	Flounder: January/February Cod: outside the breeding season.	Prior to flounder emigration from estuaries and after they are resident for maximum time period
Numbers required	At least 12	15–20	Data are usually variable, lacking normality and have uneven variance, making parametric statistical analysis of means problematic
Supporting parameters required	Fish identifier; site code and name; taxonomic identification; temperature at collection site; date of sample collection; gender; location and description of grossly visible anomalies and lesions; hepatosomatic index; gonadosomatic index; fish weight and length; age of the fish	Fish identifier; site code and name; taxonomic identification; temperature at collection site; date of sample collection; gender; location and description of grossly visible anomalies and lesions; gonadosomatic index; fish weight and length; age of the fish; histological examination of gonad to confirm sex and determine intersex occurrence and severity; bile sample for chemical analysis; liver and flesh samples for chemical analysis; liver sample for mRNA analysis	Histological examination of the gonad is useful to confirm the sex, if high Vtg is found. Intersex incidence and severity can provide information on degree of exposure to xenoestrogens in early life stages. Bile sample can be used in bioassay-directed chemical fractionation and identification of xenoestrogen metabolites. Chemical analysis will give information on known endocrine disruptors, e.g. POPs. Collection of liver samples for transcript analysis can provide additional information on the status of the animal
Sampling requirements		Staff must be trained to collect blood samples. Blood sampling must be carried out as rapidly as possible. Blood must be stored immediately on ice and centrifuged within 30 min. Plasma should be snap-frozen in liquid nitrogen. Samples must not thaw prior to assay	Vtg is a fragile protein, and erroneous results can potentially be generated by the way that the samples are handled. It is important that there is a standard operating procedure (SOP) for collection of blood samples. Repeated freezing and thawing will cause degradation of the sample. Plasma samples can be split prior to freezing and then stored in separate freezers to reduce the risk of damage
Preferred analytical method		Flounder—Vtg ELISA Cod—Lv ELISA	Flounder Vtg is relatively stable and thus the standard relatively reliable and easy to handle Cod Vtg is very unstable, Lv is a constituent protein of Vtg and can be used as a surrogate for it. For each species, a standard ELISA protocol

		and a common source of antigen and antibody are required
Assay requirements	The antibody must be specific and not cross-react with other plasma proteins; The detection limit must be below 10 ng ml^{-1}	Plasma concentrations in uninduced fish are very low. To aid subsequent statistical analysis, it is important that these concentrations are quantifiable

Vtg, vitellogenin; Lv, lipovitellin; POP, persistent organic pollutant.

Table 5.2. Plasma vitellogenin concentrations in male flounder from UK "reference" sites between 1996 and 2001, including the overall 90th percentile concentration from all "reference" sites used to set a background concentration. Also included are concentrations from the Mersey estuary for comparison

AREA	CONCENTRATION (µG ML^{-1})	*n*	RANGE OF VALUES
North Sea[a]	0.05 ± 0.01	18	<0.02–0.12
River Tyne[a]	0.04 ± 0.01	18	<0.01–0.05
River Thames site 1[a]	0.03 ± 0.003	15	<0.01–0.48
River Thames site 2[a]	0.03 ± 0.004	15	<0.01–0.59
Laboratory fish[a]	0.02 ± 0.007	15	<0.01–0.13
Pillar Bank—Clyde[b]	0.02 ± 0.002	19	<0.01–0.04
Bowling—Clyde[b]	0.08 ± 0.02	10	<0.01–0.24
Petty Roy—Clyde[b]	0.26 ± 0.22	20	<0.01–4.69
Alde reference site 1996–2001[a,b,c]		95	<0.01–0.17
Mersey estuary Dec 1996[a]	42 623 ± 15 607	25	

References: [a]Allen *et al.* (1999a); [b]Allen *et al.* (1999b); [b]Kirby *et al.* (2004a).

Table 5.3. Plasma Vtg concentrations in male cod between 2001 and 2003: concentrations in fish from reference sites and from induced fish

AREA	CONCENTRATION (µG ML^{-1})	*n*	RANGE OF VALUES
BECPELAG study (North Sea)[a]			
Males caged away from oil rig	0.04 ± 0.01	11	<0.01–0.13
Males caged near oil rig	0.24 ± 0.08	13	0.02–0.85
Norwegian cod[b]			
Males from two northern fjords	0.09 ± 0.01	22	0.04–0.25
Males from Oslofjord	3.45 ± 1.15	13	0.04–11.5
Captive males (Matre)	5.56 ± 2.67	22	0.06–46.5
Wild-caught cod in NE Atlantic[b]			
Fish <7 kg	0.17 ± 0.03	233	<0.01–4.04 (15% <assay detection limit of 0.01)
Fish >7 kg	13.14 ± 3.18	84	<0.01–159.00 (2% <assay detection limit of 0.01)
Experimentally induced[c]			
E2-implanted (after 28 d)	ca. 40 000		

Values in bold are statistically significantly higher than the other values within the same subset.

References: [a]Scott *et al.* (2006b); [b]Scott *et al.* (2006a); [c]Scott and Hylland (2002).

6 Background document: acetylcholinesterase assay as a method for assessing neurotoxic effects in aquatic organisms

Thierry Burgeot, Gilles Bocquené, Joelle Forget-Leray, Lùcia Guilhermino, Concepción Martínez-Gómez, and Kari Lehtonen

6.1 Background

The measurement of acetylcholinesterase (AChE; EC 3.1.1.7) activity in marine organisms has been shown to be a highly suitable method for assessing exposure to neurotoxic contaminants in aquatic environments. In general, the methods developed are sensitive enough to detect the neurotoxic effects of contaminant concentrations occurring in marine waters. AChE activity measurement is applicable to a wide range of species and has the advantage of detecting and quantifying exposure to neurotoxic substances without a detailed knowledge of the contaminants present. As applied in human medicine, AChE activity is a typical biomarker that can be used in *in vitro* bioassays and field applications.

AChE is present in most animals and is responsible for the rapid hydrolytic degradation of the neurotransmitter acetylcholine (ACh) into the inactive products choline and acetic acid. AChE has highest specificity for ACh of any other choline ester, whereas butyrylcholinesterase has the highest specificity for butyrylcholine or propylthiocholine. The inhibition of AChE leads to an accumulation of ACh; this, in turn, overstimulates sensitive neurons at the neuromuscular junction which results in tonic spasm and tremors. The presence of AChE has been demonstrated in a variety of tissues of marine organisms including muscle and brain tissue of fish, adductor muscle, foot tissue, haemocytes and gills of shellfish, and abdominal muscle of crustaceans (Bocquené and Galgani, 1998). The highest activities have been found in the brain and muscle of fish and in the eye and muscle of prawn (Frasco *et al*., 2010). Molluscs, in general, show low activity (Bocquené *et al*., 2004). In vertebrates, neurotoxic poisoning with hyperactivity, tremors, convulsions, and paralysis may ultimately lead to death.

Being an indicator of neurotoxic effects, AChE has traditionally been used as a specific biomarker of exposure to organophosphate and carbamate pesticides (e.g. Coppage and Braidech, 1976; Day and Scott, 1990; Bocquené and Galgani, 1998; Printes and Callaghan, 2004; Hoguet and Key, 2007). The existence of extremely low thresholds for induction of inhibitory effects on AChE suggests that detection is possible after exposure to low concentrations of neurotoxic insecticides (0.1–1 $\mu g\ l^{-1}$; Habig *et al*., 1986).

During the 1990s there was a resurgence of interest in the use of cholinesterase as a biomarker. Its responsiveness has been demonstrated to various other groups of chemicals present in the marine environment, including heavy metals, detergents, and hydrocarbons (Zinkl *et al*., 1991; Payne *et al*., 1996; Guilhermino *et al*., 1998; Forget and Bocquené, 1999; Burgeot *et al*., 2001, Brown *et al*., 2004). Its usefulness as a general indicator of pollution stress in mussels from the Baltic Sea has recently been suggested, and it has been used for this purpose (Baršienė *et al*., 2006a; Kopecka *et al*., 2006; Schiedek *et al*., 2006).

6.2 Confounding factors

It is important to know the natural limits of variability in AChE activity in the species of interest to assess the significance of any observed changes in activity.

Knowledge of possible variations related to sex, size, state of gonadal maturation, and the influence of seawater temperature should be systematically determined. Also, the presence of different cholinesterases in the same tissue having different sensitivities to anticholinesterase agents may act as a confounding factor; therefore, prior characterization of the enzymes present is recommended (Garcia *et al*., 2000). The AChE activity of juveniles of *Callionymus lyra* in the Atlantic Ocean and in *Serranus cabrilla* and *Mullus barbatus* in the Mediterranean Sea is higher than that of adults, but no differences were found between males and females in *Limanda limanda* in the Atlantic Ocean (Galgani *et al*., 1992).

Different biotic and abiotic factors are known to modulate AChE activity, including trace metals (cadmium, copper, mercury, zinc) and variations in natural environmental factors, such as seawater temperature and salinity (Leiniö and Lehtonen, 2005; Pfeifer *et al*., 2005; Rank *et al*., 2007). In *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea, mean values of AChE values vary twofold, depending on season, closely following changes in temperature (Leiniö and Lehtonen, 2005). Seasonal variability has also been shown to display different responses to natural factors in coastal areas compared with offshore sites (Dizer *et al*., 2001; Bodin *et al*., 2004; Burgeot *et al*., 2006). The presence of, and exposure to, biotoxins or cyanobacteria/cyanobacterial extracts has been demonstrated to affect AChE activity in mussels (Dailianis *et al*., 2003; Lehtonen *et al*., 2003; Frasco *et al*., 2005; Kankaanpää *et al*., 2007). Anatoxin-a(s), produced by *Anabaena flos-aquae*, is a well-known, very strong inhibitor of AChE activity. Toxins present in the water as a result of cyanobacterial blooms (e.g. *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, and *Microcystis aeruginosa*) have also been shown to inhibit AChE activity. Thus, it is recommended that the presence of any algal blooms and their identity should be noted when the samples are collected.

In crustaceans, the hormone 20-hydroxyecdysone is the primary mechanism controlling moulting and has been shown to be positively correlated with neurological activity (i.e. AChE), for example in *Artemia franciscana* (Gagne and Blaise, 2004). Moulting rate increases with development, specifically peaking at the juvenile stage. The subsequent decline in AChE may also be explained by reduced moulting frequencies in adults.

The process and mechanisms of biological response in each organism require further investigation in specific habitats with specific chemical contamination. The mussel *Mytilus galloprovincialis* shows a great heterogeneity of esterases and a particular sensitivity to specific compounds such as paraoxon (Ozretið and Krajnovið-Ozretið, 1992; Brown *et al*., 2004). The alleged versatility of AChE inhibition as an effect criterion after exposure to detergents may be misleading and may underestimate the contamination potential of complex mixtures (Rodrigues *et al*., 2011). As for many other biomarkers, hormesis effects cannot be ignored and represent a substantial scientific challenge (Kefford *et al*., 2008).

Enzymatic polymorphism has also been demonstrated in the oyster *Crassostrea gigas*, and two forms of AChE with different sensitivities to paraoxon have been described (Bocquené *et al*., 1997). Thus, extraction of the sensitive form now identified in some organisms would provide greater precision for determination of AChE enzymatic activity than would an overall measurement of acetylcholinesterases. In addition to polymorphisms, cholinesterases of some invertebrates have been shown to have different properties compared with typical forms from vertebrates. For example, cholinesterases with properties of both AChE and pseudocholinesterases have been found in the gastropods *Monodonta lineata* and *Nucella lapillus* (Cunha *et al.*, 2007), in the sea urchin *Paracenthrotus lividus* (Cunha *et al.*, 2005), in *Artemia* sp. (Varó *et al.*, 2002), and in some strains of *Daphnia magna* (Diamantino *et al.*, 2003).

Exploration of genetic variability and the influence of environmental factors in specific habitats should lead to a better distinction between natural and pollutant effects.

6.3 Ecological relevance

AChE inhibition results in continuous and excessive stimulation of nerve and muscle fibres, producing tetany, paralysis, and death. Sublethal exposure affecting AChE can alter an animal's behaviour and locomotive abilities (e.g. Vieira *et al.*, 2009), potentially affecting reproduction, fitness, and survival. Therefore, AChE should be considered an ecologically relevant parameter, potentially affecting reproduction, fitness, and survival. Evidence of modulation of AChE activity by organic chemicals, including fuel oil, has been described in marine organisms, including crustaceans (Signa *et al.*, 2008). The evaluation of the variations of AChE activity in different species allows characterization of neurotoxic effects of a wide spectrum of organic and inorganic contaminants in the marine environment.

6.4 Quality assurance

The large experience acquired in conducting AChE measurements in the field makes it possible today to evaluate the effects of diffuse contamination in some marine organisms sampled in the Atlantic Ocean, the Baltic Sea, and the Mediterranean Sea.

A microplate assay technique established for *in vitro* detection of AChE inhibition (Bocquené and Galgani, 1998) has been applied in the monitoring of coastal and offshore waters. This technique has a specific sensitivity comparable with that of chemical analyses, with a detection limit of 100 ng l^{-1} for carbamates and 10 ng l^{-1} for organophosphates (Kirby *et al.*, 2000).

Standardization of the sampling strategy and regular intercalibration exercises on specific organisms sampled in the Atlantic Ocean, Mediterranean Sea, and the Baltic Sea are necessary for the widespread use of AChE in routine pollution monitoring.

No formal quality assurance programmes are currently run within the BEQUALM programme, but one major intercalibration exercise was carried out during the BEEP project (Biological Effects of Environmental Pollution in marine coastal ecosystems, EU project EVK3-2000-00543) in 2002.

6.5 Background assessment criteria and environmental assessment criteria

Baseline levels of AChE activity in different marine species have been estimated from results derived from field studies in the Atlantic Ocean and the Mediterranean Sea (Table 6.1). Assessment criteria should be defined on a regional basis, using available long-term data.

In order to understand and apply AChE enzymatic activity as a biomarker of neurotoxic exposure it is of fundamental importance to gain information on the natural background levels in non-contaminated organisms during at least two seasonal cycles. The baseline level (35 nmol min^{-1} mg^{-1} protein) of the seasonal cycle of the mussel *Mytilus edulis* studied over three years along the Atlantic coast showed a maximum amplitude of 30% (Bocquené *et al.*, 2004).

In general, it has been accepted that 20% reduction in AChE activity in fish and invertebrates indicates exposure to neurotoxic compounds (Zinkl *et al*., 1987; Busby *et al*., 1989). Depression of AChE activity by 20–50% indicates sublethal impact (Dizer *et al*., 2001). In the field, several species have been found to have baseline AChE activities of the same order of magnitude in different studies/measurements (Table 6.1). However, differences between sea areas and seasons are obvious, for example with activity values in *Mytilus* spp. varying from 25 to 54 nmol min^{-1} mg^{-1} protein.

According to these observations, background assessment criteria (BAC) and environmental assessment criteria (EAC) were proposed using the 10th percentile of data. BAC are estimated from data from reference sites and describe the threshold values for the background level. EAC are usually derived from toxicological data and indicate a significant risk to the organism. They were calculated by subtracting 30% from the BAC values (Table 6.1) and represent a significant inhibition of AChE activity. EAC values characterize a sublethal impact. BAC and EAC should be estimated for different geographical regions and should include the effect of differences in water temperature.

Table 6.1. Assessment of acetylcholinesterase activity after *in vitro* and *in vivo* exposure of biomonitoring organisms in control laboratory conditions and field studies that have utilized common monitoring species collected from reference locations

Organism	Tissue	Reference location or control conditions	Sampling season or month	Bottom temperature or temperature range (°C)	BAC AChE 10th percentile (activity nmol min^{-1} mg^{-1} protein)	EAC (activity nmol min^{-1} mg^{-1} protein)	Reference
Invertebrates							
Mytilus galloprovincialis	Gills	Wild mussels Mediterranean Sea in Spain	May–June	15–25	15	10	J. A. Campillo-Gonzalez (pers. comm.)
Mytilus galloprovincialis	Gills	Caging in field Mediterranean Sea—Carteau, France	Seasonal cycle	14–25	29	20	Bodin *et al.* (2004)
Mytilus edulis	Gills	Wild mussels Atlantic Ocean (NW Portugal)	Seasonal cycle	-	26	19	L.Guilhermino (pers. comm.)
Mytilus edulis	Gills	Wild mussels Atlantic Ocean (Loire estuary)	Seasonal cycle	-	30	21	Bocquené *et al.* (2004)
Vertebrates							
Plathichthys flesus	Muscle	French Atlantic Ocean (Seine Bay)	-	15	235	165	Burgeot *et al.* (2001)
Plathichthys flesus	Muscle	French Atlantic Ocean (Ster estuary, Brittany)	-	15	335	235	Evrard *et al.* (2010)
Limanda limanda	Muscle	French Atlantic Ocean (Seine Bay)	-	15	150	105	Burgeot *et al.* (2001)
Mullus barbatus	Brain	Mediterranean Sea SE Spain (Màlaga-Almeria)	October	14	75	52	C. Martínez-Gómez (pers. comm.)
Mullus barbatus	Muscle	Mediterranean Sea (France, Spain, Italy)	*In situ*	18	155	109	Burgeot *et al.* (1996a); Bocquené (pers. comm.)

6.6 Future work

Standardized AChE measurement protocols and intercalibrations are required for the main species currently used in international marine biomonitoring programmes (OSPAR, HELCOM, MEDPOL, and MSFD). An *ICES TIMES* series method document has been published (Bocquené and Galgani, 1998) and can be used as the basis for standardized procedures. Further information should be gathered to confirm baseline activity levels in specific habitats and different sentinel species in Europe. The BAC and EAC values must be considered as provisional and should be updated and revised when additional relevant data become available. BAC and EAC could also be derived for new species of interest and specific local studies.

7 Background document: comet assay as a method for assessing DNA damage in aquatic organisms

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7.1 Background

The analysis of modified or damaged DNA has been shown to be a useful method for assessing exposure to genotoxic contaminants in aquatic environments. In general, the methods developed are sensitive to a range of contaminant concentrations, applicable to a wide range of species, and have the advantage of detecting and quantifying exposure to genotoxins without a detailed knowledge of the contaminants present. The single-cell gel electrophoresis (SCGE) or "comet" assay was first applied to ecotoxicology over 15 years ago, and has since become one of the most widely used tests for detecting DNA strand breaks in aquatic animals (Mitchelmore and Chipman, 1998; Cotelle and Fèrard, 1999; Lee and Steinert, 2003; Jha, 2008; Fenzilli *et al*., 2009). The comet assay has many advantages over other methods commonly used to assess genotoxic exposure, including: (i) genotoxic damage can be detected in most eukaryotic cell types at the single-cell level; (ii) only a small number of cells are required; (iii) it is a rapid and sensitive technique; (iv) owing to the nature of DNA strand-break formation, it provides an early warning response to genotoxic exposure; and (v) sites of oxidative damage can be identified using enzymatic pretreatment.

As a consequence of the advantages listed above, the comet assay has been used widely in both laboratory and field-based studies to assess genotoxic exposure in many freshwater and marine organisms. However, unlike mammalian genotoxicology, where the focus is limited to a small number of model species, efforts in the aquatic field have generally lacked coordination and have used an extensive range of sentinel species (Mitchelmore and Chipman, 1998; Lee and Steinert, 2003; Fenzilli *et al*., 2009). Although guidelines relating to the use of the comet assay have been published for mammalian genotoxicology (Tice *et al*., 2000; Burlinson *et al*., 2007), no standard protocols currently exist for environmental studies. Consequently, the variations in protocols can lead to major differences in results and an inability to directly compare studies. Despite these obvious limitations, the comet assay provides a well-researched tool for studying genotoxicity in aquatic species.

7.2 Confounding factors: protocols, cell types, and target organs

The majority of aquatic studies published to date have used circulating blood cells (either haemocytes or erythrocytes) as target cells for comet assay analysis. This is likely to be the result of the practical advantage of processing tissues from a ready-made supply of nucleated cells in suspension. Solid tissues such as gill or fish hepatocytes require dissociation prior to analysis, which has the potential to introduce damage through enzymatic or mechanical processes. Studies have also demonstrated that different cell types respond with different sensitivities to contaminant exposure. When comparing cells types, it is usually reported that circulating cells are less sensitive than hepatocytes or gill cells (Hartl *et al*., 2004; Siu *et al*., 2004; Lemiere *et al*., 2005; Kim and Hyun, 2006; Pandey *et al*., 2006; Huang *et al*., 2007). Blood and, to a lesser extent, the haemolymph of bivalve molluscs (e.g. mussels) are "buffered" tissues, in which contaminants arrive having crossed numerous biological barriers. Gill cells appeared to be the most sensitive following methylnitronitrosoguanidine exposure, whereas liver and digestive gland were more sensitive to benzo[*a*]pyrene, suggesting that uptake routes and bioaccumulation mechanisms need to be taken into account when designing experimental systems (Kim and Hyun, 2006).

Mammalian studies have demonstrated that certain tissue types may have higher background levels of DNA damage because of the presence of alkali-sensitive sites in cells with highly condensed chromatin (Singh *et al*., 1989). Similar studies comparing basal levels of DNA migration in mussel gill cells, haemocytes, and fish erythrocytes under both mild alkaline (pH 12.1) and alkaline versions (pH > 13) of comet assay have supported this assumption (Moretti *et al*., 1998; Frenzilli *et al*., 1999), indicating that the mild alkaline version of the assay should be employed when dealing with certain cell types (e.g. fish erythrocytes), in order to prevent higher background levels of DNA strand breaks inhibiting data interpretation. Indeed, this problem has been highlighted in other studies using fish species where excessive DNA tail migration has inhibited the interpretation of results (Wirzinger *et al*., 2007).

In addition to the variation in response depending on cell type, it is also apparent that a range of comet assay protocols (differing in terms of agarose concentrations, lysing, and electrophoresis parameters) have been used in studies with aquatic organisms (Mitchelmore and Chipman, 1998; Cotelle and Fèrard, 1999; Lee and Steinert, 2003; Jha, 2008; Fenzilli *et al*., 2009). Therefore, effort is required to establish standardized protocols for the main species and cell types commonly used in environmental studies. The production of standard protocols or the initiation of interlaboratory ring-testing workshops focused on aquatic species are essential if the comet assay is to develop further as an environmental monitoring tool.

A protocol has recently been developed for conserving fish erythrocytes sampled in the field for subsequent comet analysis (K. Hylland, pers. comm.), which will make the assay more directly applicable for monitoring purposes.

7.3 Ecological relevance

7.3.1 Marine invertebrates (bivalves)

Marine invertebrates have been widely used as sentinel species in environmental monitoring programmes. This is mainly the result of their ability to bioaccumulate contaminants, general ease of capture, and, for many species, their sessile nature (Bayne, 1976; Seed, 1976; Salazar and Salazar, 1995). The majority of work has focused on coastal and estuarine environments. For example, Hartl *et al*. (2004) used the clam (*Tapes semidecussatus*) as an indicator species for the presence of potentially genotoxic substances in estuarine environments, demonstrating an increase in DNA damage in haemocytes, gill, and digestive gland cells of animals exposed to contaminated sediments. The study also highlighted the differences in sensitivity between cell types, with gill and digestive gland cells appearing to be the most sensitive target tissues for detecting genotoxic exposure. The Mediterranean mussel (*Mytilus galloprovincialis*) has also been extensively deployed as a sentinel organism to assess the genotoxic effects of crude oil spills (Perez-Cadahia *et al*., 2004; Taban *et al*., 2004; Laffon *et al*., 2006). Studies have demonstrated the sensitivity of mussels to oil exposure, and laboratory studies have clearly linked the total polycyclic aromatic hydrocarbon (TPAHs) content of oils with the level of DNA damage observed (Perez-Cadahia *et al*., 2004). In northern European studies, blue mussels (*M. edulis*) have also been used to differentiate sites receiving waste treatment effluent, with positive correlations detected between the presence of selected contaminants and the level of DNA damage.

Mussels have also been used extensively in the field as part of transplantation studies (Regoli *et al*., 2004; Nigro *et al*., 2006; Rank *et al*., 2007). The use of indigenous organisms is often hampered by the absence of a suitable sentinel species, or if present, the genotoxic responses obtained may be influenced by local physiological adaptations. Furthermore, the use of transplanted organisms also offers advantages over indigenous species, such as ensuring genetic homogeneity, developmental/reproductive status, and controlling the precise exposure window. Validation studies have been undertaken with the comet assay to assess the time-course variations in DNA damage following field transplantation experiments (Regoli *et al*., 2004; Rank *et al*., 2007). It was observed that within the first 7 d following transplantation, the level of DNA damage can fluctuate, which is likely to be caused by manipulation disturbance; after 2 wk the level reaches a plateau. Such data suggest that transplantation experiments lasting less than 2 wk may give spurious results, with the levels of DNA damage detected attributable to artefacts associated with the sampling procedure rather than genotoxic exposure. Studies conducted in a coastal area of Denmark affected by a disused chemical site have also highlighted that the levels of DNA damage in mussels can be affected by seasonal variations in baseline levels (Rank *et al*., 2007). Such results are likely to be influenced by seasonal variations, which are known to exist for a range of physiological and reproductive processes in mussels (Hines *et al*., 2007; Bignell *et al*., 2008).

The sampling location has also been shown to influence the results of field-based surveys. For example, mussels (*M. edulis*) sampled from the intertidal zone in Reykjavik Harbour had higher levels of DNA damage, when compared with mussels collected from the subtidal zone at the same site (Halldórsson *et al*., 2004). Although the study supports the use of DNA strand breaks as a measure of environmental pollution, it also highlights the high levels of intrasite variability in DNA damage that can occur. As such, the study further serves to underline the importance of validating experimental protocols and sampling procedures to ensure that non-contaminant-related factors (e.g. physiological and biochemical responses to variations in oxygen availability and temperature stress) do not adversely affect biomarkers data.

7.3.2 Marine vertebrates (fish)

There are a limited number of comet assay studies utilizing marine fish species compared with those using freshwater species (for detailed review, see Mitchelmore and Chipman, 1998; Jha, 2008; Fenzilli *et al*., 2009). This is mainly because of the logistical problems associated with collecting fish at sea (e.g. need for a research vessel) and technical problems inherent within the assay, such as the difficulty of performing electrophoresis reproducibly at sea (e.g. dealing with adverse weather conditions). To date, the studies undertaken have mainly focused on flatfish and bottom-feeding species, which, owing to their close association with sediment-bound contaminants, are widely used in marine monitoring programmes (OSPAR, 1998; Feist *et al*., 2004). *In vivo* studies have been undertaken to investigate oxidative stress in the European eel (*Anguilla anguilla*; Regoli *et al*., 2003). The comet assay has also proven to be a useful tool for studying the genotoxic effects of non-bioaccumulating contaminants in the marine environment. For example, studies of the environmental effects of styrene, a known mutagen and potential carcinogen, have indicated low tendancy to bioaccumulate in the mussel (*M. edulis*) and fish (*Symphodus mellops*;

Mamaca *et al*., 2005). However, it was shown to cause a statistically significant increase in DNA damage in blood cells, probably the result of the formation of a radical styrene metabolite, which is thought to have potent oxidative capacity. Hatchery-reared turbot (*Scophthalmus maximus* L.) have been used successfully to investigate the genotoxic potential of PAH- and heavy metal-contaminated sediment from sites in Cork Harbour (Ireland; Hartl *et al*., 2007). Eelpout (*Zoarces viviparus*) have been used in site-specific investigative monitoring following a bunker oil spill in Göteborg Harbour, Sweden. The comet assay was deployed together with a battery of other bioassays, and elevated levels of DNA damage were correlated with the presence of PAH metabolites in the bile of fish (Frenzilli *et al*., 2004). The marine flatfish dab (*Limanda limanda*) is a commonly used flatfish species in offshore monitoring programmes, and it has been used in a number of studies investigating the impacts of genotoxic contaminants in coastal and estuarine waters (Akcha *et al*., 2003, 2004; Lyons *et al*., 2006). Studies have shown that both sex and age of the fish have a significant effect on the presence of DNA strand breaks, which again highlights the influence other factors (i.e. reproductive status) may have on the extent of DNA damage (Akcha *et al*., 2003, 2004).

7.4 Quality assurance

No formal quality assurance programmes are currently run within the marine monitoring community. However, a series of comet assay workshops have taken place with the aim of drafting a common regulatory strategy for industrial genotoxicology screening (Tice *et al*., 2000; Burlinson *et al*., 2007). Final guidelines drafted after the 4th International Workgroup on Genotoxicity Testing: Results of the *in vivo* comet assay workgroup (Burlinson *et al*., 2007) provide a useful starting point for developing quality assurance programmes specifically focused on protocols employed in marine species. These include consideration of (i) cell-isolation processes (if required); (ii) cryopreservation processes; (iii) concurrent measures of cytotoxicity; and (iv) image-analysis and scoring methods.

Currently, data can be reported in a number of formats. Percentage DNA in tail has been reported to be the most linearly related to exposure dose (Burlinson *et al*., 2007). However, there is no clear consensus of which measure of DNA migration should be used (% DNA in tail, tail moment, tail length). This difference in scoring criteria hinders our ability to develop a consensus background response and assessment criteria.

Members of the ICES Working Group on Biological Effects of Contaminants (WGBEC) strongly supported the development of an intercalibration exercise for comet in both blue mussel and fish. Ketil Hylland (NO) will take the initiative to generate samples for such an exercise using both types of cells. Samples will be distributed immersed in lysis buffer. This activity is currently scheduled for 2012.

7.5 Background responses and assessment criteria

It is recognized that setting baseline/background response levels has an important role in integrating biological effect parameters into environmental impact assessments of the marine environment. The general philosophy is that an elevated level of a particular biomarker, when compared with a background response, indicates that a hazardous substance has caused an unintended or unacceptable level of biological effect. Therefore, in order to understand and apply the comet assay as a biomarker of genotoxic exposure it is of fundamental importance to gain information on the natural background levels in non-contaminated organisms. Table 7.1

summarizes a number of studies that have utilized commonly deployed bioindicator species collected from reference locations (as supported by chemical and biomarker analyses) or kept under control conditions in the laboratory. Although these studies provide a starting point for determining "background" levels of DNA damage, they also serve to highlight the number of different tissues, protocols, and endpoints currently reported.

Table 7.1. Assessment of "control DNA damage" by comet assays after *in vivo* exposure to commonly used biomonitoring organisms

ORGANISM	CELL TYPE	AGENT	EXPOSURE TIME	PARAMETER	CONTROL RESPONSE	REFERENCE
Invertebrates						
M. edulis	Haemocytes	MMS	0–4 d	Tail moment	2.08 ± 3.43 2.96 ± 4.60	Rank *et al.* (2007)
M. edulis	Haemocytes	Tritiated water	96 h	% DNA tail	<10	Jha *et al.* (2005)
M. edulis	Haemocytes	TBT	7 d	% DNA tail	5–10	Hagger *et al.* (2005)
M. edulis	Haemocytes	MMS	3–7 d	% DNA tail	<10	Canty *et al.* (2009)
M. edulis	Gill cells	Cd Cr Cr VI	10 d 7 d injection	% DNA tail	<15	Emmanouil *et al.* (2006)
M. edulis	Gill cells	MMS		Tail moment	1.87 ± 2.23 0.60 ± 1.05 3.84 ± 3.61 1.22 ± 1.47	Rank *et al.* (2007)
M. edulis	Gill cells	Field site	*In situ*	Tail moment	<1.5	Rank (2009)
M. edulis	Gill cells	Field site	*In situ*	Tail moment	<5	Rank *et al.* (2007)
M. edulis	Digestive gland	H_2O_2, BaP	1 h	% DNA tail	<10	Mitchelmore *et al.* (1998)
Vertebrates						
L. limanda	Erythrocytes	Field	*In situ*	Tail moment	<5	Lyons *et al.* (2006)
L. limanda	Erythrocytes	Field	*In situ*	% DNA tail	4–6	Akcha *et al.* (2003)
P. olivaceus	Erythrocytes	Field	*In situ*	Tail length (μm)	<0	Woo *et al.* (2006)
Z. viviparus	Erythrocytes	Field	*In situ*	% DNA tail	<5	Frenzilli *et al.* (2004)

In addition to the above, there was a recent study as part of the Integrated Asessment of Contaminant Impacts on the North Sea (ICON) in which dab (*Limanda limanda*) were collected from the North Sea and in Icelandic waters (J. Skei, pers. comm.). Ninety percentiles from the reference location support a value of 4–5% tail DNA as a BAC assessment criterion for this species.

In laboratory experiments with Atlantic cod (*Gadus morhua*), a comet assay value of 4.9% tail DNA was measured in the control group (S. Sanni, pers. comm.). The water was supplied continuously from a non-polluted source at 78 m depth at a North Sea coastal location outside Stavanger, Norway. In this experiment, dose-dependent increases in comet values were observed with increasing exposure concentrations of produced water, but the range of concentrations in the study were not large enough to be able to establish EAC comet values corresponding to critical mortality values for larval stages of cod. At the highest exposure in the experiment, the comet value was 8.4% tail DNA, hence the EAC comet value (based on toxicity experiments) can be expected to be at a higher level than this.

In a similar experiment with blue mussel (*Mytulis edulis*) haemocytes, the comet value in the controls was 7% tail DNA, whereas the comet value corresponding to the exposure level of a dispersed North Sea crude oil critical for mussel larval mortality was 14% tail DNA (Baussant *et al*., 2009).

From the above, it would appear that a preliminary BAC for comet analyses of dab and Atlantic cod erythrocytes could be set at 5%. There are not sufficient data to provide EAC at this time.

For mussel haemocytes, available data suggest a BAC of 10%. One study has been able to determine an effect level that could be used to derive EAC (14%), but this needs to be supported by further studies.

There is a requirement for a standardized protocol for the main species used in monitoring programmes (dab, flounder, cod, blue mussel), including minimum acceptable reporting criteria (cellular toxicity, ± control, etc.) and a decision about reporting format (tail moment, % DNA in tail). There is, furthermore, a need for quality assurance (QA) and intercalibration exercises (will be initiated by WGBEC members) and further evaluation of the suggested assessment criteria.

8 Background document: DNA adducts of polycyclic aromatic hydrocarbons

Brett Lyons and Ian M. Davies

8.1 Background

In the chemical carcinogenesis model, the initiating step is the covalent modification of DNA by a carcinogen (Miller and Miller, 1981). The measurement of covalent structures formed between environmental carcinogens and DNA, termed DNA adducts, can be utilized as a biological marker of exposure to genotoxic compounds. DNA adducts can be removed by cellular repair processes or by cell death, but during chronic exposures, they often reach steady-state concentrations in carcinogen target tissues, such as the liver. As a consequence, DNA adducts have several important features which make them suitable as biomarkers of carcinogen exposure:

- It is a quantifiable measurement of the biologically effective dose of a contaminant reaching a critical cellular target and, therefore, a useful epidemiological biomarker for detecting exposure to environmental genotoxins.
- DNA adduct levels integrate multiple toxicokinetic factors, such as uptake, metabolisms, detoxification, excretion, and DNA repair in target tissues.
- DNA adducts are relatively persistent once formed (may last several months); therefore, they provide an assessment of chronic exposure accumulated over many weeks rather than a few days, as afforded by other PAH biomarkers such as EROD induction or the presence of bile metabolites.
- Studies from North America have demonstrated that risk factors for certain lesions can be generated by correlating the level of DNA damage with lesion occurrence, thus allowing the use of a relatively simple biomarker in predicting risk.

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous and large group of environmental contaminants, some of which are known to cause genetic toxicity through the formation of DNA adducts. Over the past 25 years, a growing body of research has investigated the uptake, bioaccumulation, and metabolism of PAHs, and there is now extensive experimental and field-based evidence supporting their role in the initiation and progression of chemical carcinogenesis. Numerous field studies in both North America and Europe have established a correlation between PAH sediment concentrations and the prevalence of hepatic tumours in fish (Malins *et al*., 1985; Myers *et al*., 1991; Baumann, 1998). For example, liver and skin neoplasia in brown bullheads (*Ictalurus nebulosus*) from the Black River, Ohio (USA) have been shown to be strongly correlated with PAH sediment contamination (Baumann, 1998). Further work carried out in Puget Sound (USA) has also found positive correlations between hepatic lesions, including neoplasia (hepatocellular carcinomas and cholangiocellular carcinomas) and foci of cellular alteration (pre-neoplastic lesions) in English sole (*Parophrys vetulus*) and sediment PAH contamination (Malins *et al*., 1985). Therefore, the measurement of DNA adduct levels in marine organisms is an important step in assessing risk from exposure to environmental carcinogens and mutagens.

Of the techniques currently available for the detection of DNA adducts, the most sensitive method for the detection of a wide range of compounds chemically bound to DNA is the ^{32}P-postlabelling assay (Gupta *et al.*, 1982). The method possesses a number of advantages that make it suitable for the assessment of DNA adducts induced by environmental genotoxins (for a review, see Beach and Gupta, 1992; Phillips, 1997, 2005). The technique is applicable to any tissue sample from which DNA can be isolated, and is also extremely sensitive, capable of detecting one adducted nucleotide in 10^9–10^{10} undamaged nucleotides from 5–10 μg DNA. In addition, providing the adduct is amenable to the labelling reaction and subsequent thin-layer chromatography, its prior characterization is not required. It is this last feature that makes the assay particularly appropriate to aquatic biomonitoring, because it is suitable for the analysis of the diverse array of adducts induced by complex mixtures of environmental chemicals. It is important to note that ^{32}P-postlabelling is only semi-quantitative, as not all DNA adducts are labelled with the same efficiency, and the various enrichment and chromatograph steps involved will preferentially select certain adducts. However, the assay's sensitivity, coupled with its ability to detect a wide range of carcinogens (e.g. PAHs), has led to its widespread use in environmental biomonitoring programmes using both vertebrate and invertebrate sentinel organisms (Van der Oost *et al.*, 1994; Ericson *et al.*, 1998; Lyons *et al.*, 1999; Akcha *et al.*, 2004; Lyons *et al.*, 2004a; Balk *et al.*, 2006), following exposure to specific environmental genotoxins (Ericson *et al.*, 1999; Lyons *et al.*, 1999) and to compounds present in organic extracts from PAH-contaminated sediments (Stein *et al.*, 1990; French *et al.*, 1996).

8.2 Ecological relevance and validation for use in the field

The field validation of a biomarker of exposure, such as DNA adducts, is essential in establishing its credentials when used in routine monitoring programmes. In North America the technique has been widely used (>30 marine and freshwater species), and guidelines for implementation are published in an *ICES TIMES* technical document (Reichert *et al.*, 1999). Across the OSPAR maritime area, the assay has been used in several biological effects monitoring programmes using a range of indicator species including blue mussels (*Mytilus* sp.), perch (*Perca fluviatilis*), dab (*Limanda limanda*), European flounder (*Platichthys flesus*), eelpout (*Zoarces viviparous*), and cod (*Gadus morhua*; Ericson *et al.*, 1998, 2002; Lyons *et al.*, 1999, 2000, 2004a,b; Aas *et al.*, 2003; Akcha *et al.*, 2004; Balk *et al.*, 2006). Studies from both North America and Europe have clearly demonstrated that, when using non-migratory fish, the levels of DNA adducts strongly correlate with the concentration of PAH sediment contamination (Van der Oost *et al.*, 1994; Ericson *et al.*, 1999; Lyons *et al.*, 1999). For example, studies using the eel (*Anguilla anguilla*) demonstrated a significant relationship between the level of DNA adducts and PAH contamination of the sediment (Van der Oost *et al.*, 1994). Laboratory studies have demonstrated that fish exposed to PAHs accumulate hepatic DNA adducts in both a time- and a dose-dependent manner (French *et al.*, 1996). It is known from experimental studies using both fish and shellfish that such DNA adducts may persist for many months once formed and are, therefore, particularly suited to monitoring chronic exposure to genotoxic contaminants (Stein *et al.*, 1990; French *et al.*, 1996; Harvey and Parry, 1998). Significantly, field-based studies have investigated the relationship between DNA adduct formation and neoplastic liver disease, and it has been demonstrated that, at certain contaminated sites, the prevalence of DNA adducts is associated with the prevalence of toxicopathetic lesions, including foci of cellular alteration and neoplasia (for review, see Reichert *et al.*, 1998).

Studies from North America and Europe suggest that DNA adduct levels are not markedly influenced by factors such as age, sex, season, or dietary status, which are known to confound the interpretation of other biomarkers (e.g. EROD). However, validation of any biomarker, including DNA adducts in a species of interest, is essential to ensure against any unforeseen species-specific responses (Reichert *et al.*, 1999). Although there is no evidence to suggest that environmental factors such as salinity and temperature significantly affect the formation of DNA adducts, these factors should always be considered, as it is known that cellular detoxification systems (e.g. CYP1A) are influenced by changes in environmental variables (Sleiderink *et al.*, 1995b).

8.3 Species selection and target tissue

The majority of hydrophobic genotoxins, such as PAHs, released into the marine environment quickly adhere to organic particular matter and settle into the sediment. Therefore, the majority of fish species used in PAH contaminant monitoring programmes are benthic feeders, such as the marine flatfish. A particular advantage of the ^{32}P-postlabelling assay is that it is not species-specific and, therefore, can be used on any organism deemed fit for this purpose. As such, it has been used widely in a range of species (both vertebrate and invertebrate), ranging from filter-feeders to high-order predators. It should be noted that DNA adducts are known to accumulate and persist over time (Stein *et al.*, 1990; French *et al.*, 1996), and consequently should be considered a cumulative index, integrating both past and present genotoxic exposure. Therefore, care needs to be taken when undertaking studies in migratory fish species as the detectable levels of DNA adducts may not be a true representation of the genotoxic contaminants at the site of capture. It has been suggested by Reichert *et al.* (1999) that, in such situations, biomarkers such as bile metabolite analysis should be employed in parallel, as this would provide a relatively accurate index of recent PAH exposure and would, therefore, indicate whether or not the levels of DNA adducts were the result of exposure at the site of capture.

Of the affected organs, liver is the most commonly studied when fish are used as sentinel organisms. Field data infer a chemical aetiology for many of the commonly observed hepatic lesions seen in wild fish collected from contaminated areas. Laboratory data supporting this association stem from biochemical and molecular studies which have revealed the liver to be the major site for contaminant detoxification pathways (e.g. cytochrome P450-mediated biotransformation enzyme systems). Furthermore, contaminant metabolisms studies have demonstrated fish liver microsomes are capable of producing the ultimate carcinogenic forms of common environmentally relevant PAHs, including benzo[*a*]pyrene, which bind to DNA to form adducts (Sikka *et al.*, 1991). As mentioned previously, a major strength of the ^{32}P-postlabelling assay is that it is not tissue-specific; assuming sufficient DNA can be extracted, it can be applied in a fit-for-purpose manner in any tissue of choice. To this end, it has been used successfully in a range of tissues (both invertebrate and vertebrate), including liver, intestine, gill, brain, gonad, and digestive gland (French *et al.*, 1996; Lyons *et al.*, 1997; Harvey and Parry, 1998; Ericson *et al.*, 1999).

8.4 Methodology and technical considerations

In the ^{32}P-postlabelling method, DNA isolated from tissue is first hydrolysed enzymatically to 3′-monophosphates. The proportion of adducts in the enzyme hydrolysate is enriched by selective removal of unmodified nucleotides by enzymatic methods (Reddy and Randerath, 1986) or by extracting the adducts into *n*-

butanol (Gupta, 1985) before labelling the mononucleotides with ^{32}P-ATP. For hydrophobic aromatic DNA adducts, such as PAH–DNA adducts, the enrichment steps can enhance the sensitivity of the assay to detect one adduct in 10^{9}–10^{10} bases (Reichert *et al*., 1999). Following the adduct-enrichment step, the 3′-monophosphates are radiolabelled at the 5′-hydroxyl using ^{32}P-ATP and T4-polynucleotide kinase to form 3′, (^{32}P)5′-bisphosphates. Separation of the ^{32}P-labelled adducts is accomplished by multidimensional, high-resolution, anion-exchange, thin-layer chromatography. Autoradiography is then used to locate the radiolabelled adducts on the chromatogram, and the radioactivity is measured by either liquid scintillation spectroscopy or storage-phosphor imaging (IARC, 1993; Phillips and Castegnaro, 1999). Detailed methodologies have which have been through appropriate quality assurance (QA) programmes are now published by ICES and IARC (Phillips and Castegnaro, 1999; Reichert *et al*., 1999).

8.5 Radiation safety

The ^{32}P-postlabelling assay uses large amounts of ^{32}P, which is an energetic beta emitter (1.7 MeV) with a half-life of 14.3 d. Researchers using this isotope must receive detailed instruction before handling ^{32}P and must be frequently monitored for exposure to ^{32}P. In the UK, the use of ^{32}P in scientific procedures is governed by the Environment Agency. Institutes need to have an appointed radiation protection supervisor (RPS) and follow designated licence-consent criteria. Institutes wishing to conduct ^{32}P-postlabelling outside the UK must contact their own national licensing organization to clarify the legislative procedures required.

Main considerations to help minimize and monitor ^{32}P exposure:

- All researchers who handle ^{32}P must wear a whole-body film badge and a finger dosimeter on the inside of each hand where there is the highest potential for radiation exposure. These badges should be monitored regularly.
- All laboratory operations are planned to minimize the time spent handling radioactivity, and the use of tongs and forceps to minimize handling of tubes and vials is recommended.
- Double-latex gloves are worn while handling ^{32}P and should be regularly checked for radioactivity by passing them under a radiation monitor. Gloves should immediately be changed and discarded if contaminated.
- Laboratory working surfaces are checked frequently with the radiation monitor when handling ^{32}P. The monitor probe should be covered with a thin vinyl wrap to prevent contamination of the detector.
- After completion of work with radioactivity, workers are to check themselves and their equipment with the radiation monitor. If any radioactivity is detected, they are to wash themselves and/or the equipment until free of radioactivity.

8.6 Equipment for handling and storage of ^{32}P

All ^{32}P is handled behind 1 cm Perspex/Plexiglas shielding. In addition, samples are kept in Perspex/Plexiglas containers that are at least 1 cm thick. Where possible, all manipulations of eppendorfs and vials should be conducted using long-armed tongs. It is recommended that radioactive waste is temporarily stored in a 1-cm-thick Perspex/Plexiglas box. Such radiation-specific safety equipment is available from most large scientific suppliers. Researchers should ensure that all safety procedures comply implicitly with their local radiation protection regulations. Detailed laboratory safety procedures are discussed in further in Castegnaro *et al*. (1993).

8.7 Status of quality control procedures and standardized assays

There are currently no active QA programmes running for the detection of DNA adducts using the ^{32}P-postlabelling method. Previous QA programmes have been conducted under the auspices of the EU-funded Biological Effects Quality Assurance in Monitoring Programme (BEQUALM) and the International Agency for Research on Cancer (IARC). The IARC QA trial of the ^{32}P-postlabelling assay was conducted between 1994 and 1997 and involved 25 participants in Europe and the USA. The primary objectives of this project were to standardize the ^{32}P-postlabelling assay and improve interlaboratory reproducibility. The IARC QA programme for ^{32}P-postlabelling led to a series of publications, which detailed a standardized protocol for the detection of bulky aromatic DNA adducts by the ^{32}P-postlabelling assay (IARC, 1993; Phillips and Castegnaro, 1999). The standardized protocol has now been adopted by the International Programme on Chemical Safety (IPCS)[1] and recommended for use in their guidelines for monitoring genotoxic carcinogens in humans (Richard *et al*., 2000). Essentially the same protocol is also published in an *ICES TIMES* technical document (Reichert *et al*., 1999).

8.8 Assessment criteria

It is recognized that baseline/background response levels have an important role in integrating biological effect parameters into environmental impact assessments of the marine environment. The general philosophy is that an elevated level of a particular biomarker, when compared with a background response, indicates that a hazardous substance has caused an unintended or unacceptable level of biological effect. Therefore, in order to understand and apply DNA adducts as biomarkers of genotoxic exposure, it is of fundamental importance to gain information on the natural background levels in non-contaminated organisms. A number of studies have now examined fish collected from pristine areas (as supported by chemical and biomarker analyses), and the typical ^{32}P-postlabelling-generated DNA adduct profiles either exhibited no detectable adducts or very faint diagonal radioactive zones (DRZs; Figure 8.1A), suggesting minimal PAH exposure (Ericson *et al*., 1998; Reichert *et al*., 1998; Lyons *et al*., 2000; Aas, *et al*., 2003; Balk *et al*., 2006). In contrast, DNA adduct profiles in fish exposed to a complex mixture of PAHs will form DRZs on the chromatogram (Figure 8.1B), which is a composite of multiple overlapping PAH–DNA adducts.

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1 International Programme on Chemical Safety (IPCS) was established in 1980 under the WHO, for more information visit: http://www.who.int/ipcs/en/

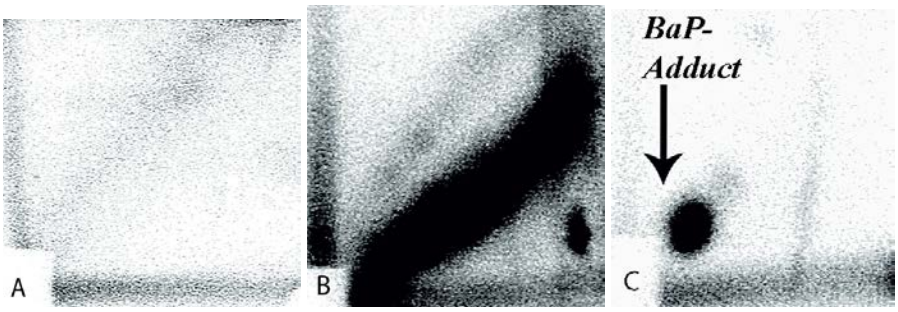

Figure 8.1. Representative hepatic DNA adducts profiles produced following ^{32}P-postlabelling. (A) DNA adduct profile obtained from a site with a low level of PAH contamination. A faint DRZ is visible, indicating a low level of DNA adducts representative of a clean reference location. (B) DNA adduct profile displaying a clear DRZ of ^{32}P-labelled DNA adducts, indicating the fish has been exposed to a complex mixture of genotoxins. (C) Positive control consisting of benzo[*a*]pyrene-labelled DNA (115 nucleotides 10^{-8} undamaged nucleotides) run with each batch (kindly provided by Professor David Phillips and Dr Alan Hewer, Cancer Research Institute, Sutton UK). Figure adapted from Lyons *et al.* (2004b).

8.9 Determination of threshold level of significant effects for DNA adducts in cod

The determined 90th percentile background level for DNA adducts in cod can be used to express the elevated-above-background level; however, this level is not associated with significant effects on fitness in whole organisms. Therefore, we have also defined a threshold value of significant effects. This is achieved by combining fitness-effect data with DNA adduct data at corresponding oil concentrations.

Dose–response relationships between exposure concentrations of oil and DNA adducts in cod have been established in laboratory studies. We have used data from Skadsheim (2004) and Skadsheim *et al.* (2009). Determination of significant whole-organism effects on fitness is more uncertain. We have here assumed that this threshold level is found between 0.5 and 1.0 ppm of oil. We base this on reproduction-effect data in model fish species *Cyprinodon variegatus* exposed to oil (Anderson *et al.*, 1977). These data have later been included in generic species-sensitivity distribution for chronic whole-organism effects (Scholten *et al.*, 1993; Smit *et al.*, 2009). This corresponds to mortality levels found in larval studies with the Northeast Atlantic-relevant species herring and halibut exposed to oil (A. Ingvarsdottir, pers. comm.).

Within the concentration range of 0.5–1.0 ppm oil, DNA adduct formation tends to increase strongly (Skadsheim *et al.*, 2009). The interpolated DNA adduct value at mid-range (0.75 ppm oil) was 6 nmol adducts mol^{-1} nucleotides. A similar value has also been found for turbot at this oil concentration (G. Jonsson, pers. comm.). This value may be revised as new data emerge to determine chronic effect levels in cod.

The following issues are important and require consideration:

- ^{32}P-postlabelling studies should be conducted using internationally agreed protocols incorporating appropriate positive and negative control samples (Phillips and Castegnaro, 1999; Reichert *et al.*, 1999).
- All studies need to include supporting environmental data to confirm the contaminant load at the reference location and, where possible, supporting biomarker and histopathological data to confirm health status of the individual.

- Although the ^{32}P-postlabelling assay can be applied to any species deemed fit for purpose, it should only be applied to species where there is sufficient background information available on life-history traits and behaviour (e.g. migration).

8.10 Derivation of assessment criteria

The UK has monitored DNA adducts in dab at offshore locations at 15 sites and for flounder in eight estuaries. Using these studies, it has been possible to define reference locations and develop background response ranges. The approach used is similar to that adopted by the US EPA on effect-range (ER) values. The ER-low (ER-L) value is defined as the lower 10th percentile of the effect. Data were available from Norway (IRIS and NIVA) for other species (IRIS database; BioSea project—Total E and P Norge and Eni Norge); data were reported as nmol adducts mol^{-1} DNA. The UK expressed results as adducted nucleotides per 10^8 normal nucleotides, which was converted to nmol adducts mol^{-1} DNA by dividing by 10.

The derived values were ER-L 1.0 (background) for dab and flounder, 1.6 (background) for Atlantic cod, and 3.0 (subtracting a species-specific spot) for haddock (Barents Sea). Threshold value assigned for significant effects in Atlantic cod was 6 (see above for method of estimation). This value is also indicative for flatfish (to be verified).

Table 8.1. Summary of assessment criteria.

Biological effect	Qualifying comments	Background response range	Elevated response range	High and cause-for-concern response
DNA adducts; nm adducts mol^{-1} DNA	Dab	≤1.0	>1.0	>6
	Flounder	≤1.0	>1.0	>6
	Cod	≤1.6	>1.6	>6
	Haddock	≤3.0	>3.0	>6

8.11 Concluding remarks

- **DNA adducts as biomarkers of genotoxic exposure.** DNA adducts provide a measure of the biologically active contaminants to have reached a critical cellular target (DNA). They are persistent and, therefore, considered a "cumulative index" of exposure to genotoxins, and a significant body of research demonstrates their importance in the initiation and progression of carcinogenesis induced by important environmental contaminants (e.g. PAHs).
- **Safety considerations when conducting the ^{32}P-postlabelling assay.** The ^{32}P-postlabelling assay uses large amounts of ^{32}P, which is an energetic beta emitter. This requires specialist laboratories, which may limit the use of the assay to a few appropriately equipped research groups.
- **Applicability across OSPAR maritime area.** DNA adducts have been applied in a wide range of species across the whole OSPAR maritime area, including blue mussels (*Mytilus* sp.), perch (*Perca fluviatilis*), dab (*Limanda limanda*), European flounder (*Platichthys flesus*), eelpout (*Zoarces viviparous*), and cod (*Gadus morhua*). A particular advantage of the ^{32}P-postlabelling assay is that it is not species-specific and, therefore, can be utilized on any organism deemed fit for purpose.

- **Status of quality assurance.** There are currently no active QA programmes running for the detection of DNA adducts using the ^{32}P-postlabelling method. However, interlaboratory QA programmes have previously been conducted under the auspices of BEQUALM and IARC, and standardized protocols are available in the form of an *ICES TIMES* technical document and IARC publications.
- **Assessment criteria.** Provisional assessment criteria have been derived for flounder, dab, and Atlantic cod. In addition, background criteria have been set for haddock and long rough dab (*Hippoglossoides platessoides*). These have been derived from datasets from national monitoring programmes within the OSPAR maritime area. It is recommended that further work to refine these values is taken forward as and when new data become available through national monitoring programmes and through the activities of ICES WGBEC.

9 Background document: lysosomal stability as a global health status indicator in biomonitoring

Mike N. Moore

9.1 Background

Lysosomal functional integrity is a generic common target for environmental stressors in all eukaryotic organisms, from yeast and protozoans to humans (Cuervo, 2004), that is evolutionarily highly conserved. Lysosomal membrane stability is a good diagnostic biomarker of individual health status (Moore, 1976, 1985, 1988, 1990, 2002; Lowe *et al.*, 1981, 1992, 1995a, 2006; Lowe, 1988; Köhler *et al.*, 1992, 2002; Cajaraville *et al.*, 1995, 2000; Svendsen and Weeks, 1995; Bayne and Moore, 1998; Klionsky and Emr, 2000; Lekube *et al.*, 2000; Burlando *et al.*, 2002; Galloway *et al.*, 2002, 2004; Winston *et al.*, 2002; Marigomez and Baybay-Villacorta, 2003; Allen and Moore, 2004; Hankard *et al.*, 2004; Moore *et al.*, 2004a, 2006a,b,c; Svendsen *et al.*, 2004; Nicholson and Lam, 2005; Dondero *et al.*, 2006). Dysfunction of lysosomal processes has been mechanistically linked with many aspects of pathology associated with toxicity and degenerative diseases (Köhler *et al.*, 2002; Cuervo, 2004; Köhler *et al.*, 2004; Moore *et al.*, 2006a,b). Recent studies have shown that lysosomal autophagy provides a second line of defence against oxidative stress (Cuervo, 2004; Moore *et al.*, 2006c), and the capacity to effectively up-regulate this process is probably a significant factor contributing to the ability of some organisms to tolerate stressful and polluted environments.

Lysosomal membrane stability has been adopted by the UN Environment Programme (UNEP) as part of the first tier of techniques for assessing harmful impact in the Mediterranean Pollution programme (MED POL Phase IV). Other lysosomal biomarkers, including lipofuscin in molluscs (age/stress pigment) and lysosomal neutral lipid (chemically induced lipidosis) in molluscs and fish, have been adopted as part of the second tier assessment methods (Moore, 1988; Krishnakumar *et al.*, 1994; Moore *et al.*, 2004b).

This biomarker can also be used prognostically to predict liver damage and tumour progression in the liver of various fish species (Broeg *et al.*, 1999a,b; Köhler *et al.*, 2002, 2004), and hepatopancreatic degeneration in molluscs (e.g. blue and green mussels, freshwater bivalves and snails, periwinkles, oysters), coelomocyte damage in earthworms, as well as enhanced protein turnover (i.e. lysosomal autophagy) as a result of radical attack on proteins; and energetic status (i.e. scope for growth) as a predictive indicator of fitness of individuals within a population (Kirchin *et al.*, 1992; Svendsen and Weeks, 1995; Köhler *et al.*, 2002; Allen and Moore, 2004; Moore *et al.*, 2004b, 2006a; Svendsen *et al.*, 2004; Nicholson and Lam, 2005).

Lysosomes are known to accumulate many metals and organic xenobiotics. Adverse lysosomal reactions to xenobiotic pollutants include swelling, lipidosis (pathological accumulation of lipid), lipofuscinosis (pathological accumulation of age/stress pigment) in molluscs, but not fish, and loss of membrane integrity (Viarengo *et al.*, 1985a; Moore, 1988; Köhler *et al.* 2002; Moore *et al.*, 2006a,b). Metals such as copper, cadmium, and mercury will also induce lysosomal destabilization in mussels (Viarengo *et al.*, 1981, 1985a,b), and if oxyradicals are generated, then lipofuscinosis can also occur (Viarengo *et al.*, 1985b).

Lysosomal membrane integrity or stability in blue mussels is correlated with oxygen and nitrogen radical-scavenging capacity (TOSC), protein synthesis, scope for growth, and larval viability (oysters—*Crassostrea gigas*); and inversely correlated with DNA damage (incidence of micronuclei), lysosomal swelling, lipidosis, and lipofuscinosis, which are characteristic of failed or incomplete autophagy (Krishnakumar *et al*., 1994; Regoli, 2000; Dailianis *et al*., 2003; Kalpaxis *et al*., 2004; Moore *et al*., 2004a,b, 2006a; Ringwood *et al*., 2004). In fish liver, lysosomal membrane stability is strongly correlated with suppression of the activity of macrophage aggregates (Broeg, 2003; Broeg *et al*., 2005), lipidosis, and lipofuscinosis (Broeg *et al*., 1999a,b; Köhler *et al*., 2004; K. Broeg, pers. comm.).

Lysosomal stability and other lysosomal biomarkers, such as lipofuscin, are strongly correlated with mussel tissue concentration of polycyclic aromatic hydrocarbons (PAHs), which are ubiquitous contaminants (Moore, 1990; Viarengo *et al*., 1992; Krishnakumar *et al*., 1994; Cajaraville *et al*., 2000; Moore *et al*., 2006a,b,c), as well as organochlorines and PCB congeners in liver of fish (Köhler *et al*., 2002). Lysosomal stability of various species of mussel and fish from different climate zones clearly reflects gradients of complex mixtures of chemicals in water and sediments (Da Ros *et al*., 2002; Pisoni *et al.,* 2004; Sturve *et al*., 2005; Baršienė *et al*., 2006a; Schiedek *et al*., 2006), single pollution events and accidents (Broeg *et al*., 2002; Einsporn *et al*. 2005; Nicholson and Lam, 2005), and also serves for the discovery of new "hot spots" of pollution (Moore *et al*., 1997, 1998a,b; 2004b; Bressling, 2006).

A conceptual mechanistic model has been developed linking lysosomal damage and autophagic dysfunction with injury to cells, tissues, and the whole animal. The complementary use of a cell-based bioenergetic computational model of molluscan hepatopancreatic cells that simulates lysosomal and cellular reactions to pollutants has also been demonstrated (Lowe, 1988; Allen and McVeigh, 2004; McVeigh *et al*., 2006; Moore *et al*., 2006a,b,c). The integration of biomarker data can be achieved using multivariate statistics and then mapped onto a two-dimensional representation of "health-status space" (see below) by using lysosomal membrane stability as a measure of cellular well-being (Lowe, 1988; Moore, 1988; Clarke, 1999; Allen and Moore, 2004; Dondero *et al*., 2006; Moore *et al*., 2006a; Dagnino *et al*., 2007). This is viewed as a crucial step towards the derivation of explanatory frameworks for prediction of pollutant impact on animal health.

Health-status space is analogous to phase space in physics. For a system of n first-order ordinary differential equations, the $2n$-dimensional space consisting of the possible values of x is known as its phase space. In its simplest form, it is a two-dimensional graph where any point can be described in terms of two numbers, the x and y coordinates. The dimensions of multidimensional health-status space are multiple contaminant and biomarker data, environmental variability, space, and time. Principal component analysis (PCA) has been used to reduce the dimensionality of the problem to a simple two-dimensional representation (Allen and Moore, 2004; Lowe *et al*., 2006; Moore *et al*., 2006a).

9.2 Confounding factors

Lysosomal stability is an indicator of health status and will be affected by non-contaminant factors, such as severe nutritional deprivation, severe hyperthermia, and prolonged hypoxia (Moore *et al*., 1980, 2007). Processing for neutral red retention (NRR) in samples of molluscs should use either physiological saline adjusted to the equivalent ionic strength or else use ambient-filtered seawater. The major confounding factor in respect of biomonitoring is the adverse effect of the final stage of gametogenesis and spawning in mussel, which is a naturally stressful process (Bayne and Widdows, 1978). In general, this period should be avoided for sampling purposes because most physiological processes and related biomarkers are adversely affected (Moore *et al.*, 2004b). However, for fish, spawning has only a minimal effect on lysosomal stability and does not mask harmful chemical-induced damage to lysosomal membrane stability (Köhler, 1991).

9.3 Ecological relevance

Lysosomal integrity is directly correlated with physiological scope for growth (SFG) and is also mechanistically linked in terms of the processes of protein turnover (Allen and Moore, 2004; Moore *et al.*, 2006a). Ringwood *et al.* (2004) have also shown that lysosomal stability in parent oysters is directly correlated with larval viability. Finally, lysosomal stability is also directly correlated with diversity of macrobenthic organisms in an investigation in Langesund Fjord in Norway (Moore *et al.*, 2006b).

9.4 Quality assurance

Intercalibration exercises for lysosomal stability techniques have been carried out in the ICES/UNESCO-IOC-GEEP Bremerhaven Research Workshop and UNEP-MED POL programme, and for the NRR method in the GEF Black Sea Environment Programme (Köhler *et al.*, 1992; Lowe *et al.*, 1992; Moore *et al.*, 1997, 1998a,b; Viarengo *et al.*, 2000). The results from these operations indicated that both techniques could be used in the participating laboratories in an effective manner with insignificant interlaboratory variability.

The standards used in this intercalibration involved digestive glands from marine mussels prepared at the University of Genova / University of Eastern Piedmont, Alessandria (Italy). Comparisons of the cytochemical and the NRR techniques have been performed in fish liver (ICES-IOC Bremerhaven Workshop, 1990) and in mussels experimentally exposed to PAHs (Lowe *et al.*, 1995b).

A workshop (ICES/OSPAR WKLYS) on the quality and interpretation of lysosomal stability data was conducted in 2010 (ICES, 2010a). The first intercalibration exercise using NRR assay was carried out in 2009 and linked 16 MED POL and ICES laboratories,. Results were presented in the Consultation Meeting to review MED POL in 2011 (UNEP/MAP, 2012).

9.5 Background responses and assessment criteria

Health-status thresholds for NRR and cytochemical methods for lysosomal stability have been determined from *Mytilus* sp. data based on numerous studies performed in the UK (Cajaraville *et al.*, 2000; Moore *et al.*, 2006a).

Lysosomal stability is a biophysical property of the bounding membrane of secondary lysosomes and appears to be largely independent of taxa. In all organisms tested to date, which includes protozoans, annelids (terrestrial and marine), molluscs (freshwater and marine), crustaceans (terrestrial and aquatic), echinoderms, and fish, the absolute values for measurement of lysosomal stability (NRR and cytochemical method) are directly comparable. Furthermore, measurements of this biomarker in animals from climatically and physically diverse terrestrial and aquatic ecosystems also indicate that it is potentially a universal indicator of health status. For the cytochemical method, animals are considered to be healthy if the lysosomal stability is >20 min; stressed, but compensating if <20 but >10 min and severely stressed and probably exhibiting pathology if <10 min (Moore *et al.*, 2006a). Similarly for the NRR method, animals are considered to be healthy if NRR is >120 min; stressed, but compensating if <120 but >50 min and severely stressed and probably exhibiting pathology if <50 min (Moore *et al.*, 2006a).

10 Background document: micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms

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10.1 Background

Micronuclei (MN) consist of acentric fragments of chromosomes or whole chromosomes that are not incorporated into daughter nuclei at anaphase. These small nuclei can be formed as a consequence of the lagging of a whole chromosome (aneugenic event) or acentric chromosome fragments (clastogenic event; Heddle, 1973; Schmid, 1975). A micronucleus (MN) arises in cell divisions as a result of spindle-apparatus malfunction, the lack of or damage to a centromere, or chromosomal aberrations (Fenech, 2000).

Clastogens induce MN by breaking the double helix of DNA, thereby forming acentric fragments that are unable to adhere to the spindle fibres and integrate in the daughter nuclei, and are thus left out during mitosis. Aneuploidogenic agents are chemicals that prevent the formation of the spindle apparatus during mitosis. This leads to the generation of not only whole chromatids that are left out of the nuclei, thus forming MN, but also multinucleated cells in which each nucleus contains a different number of chromosomes (Serrano-García and Montero-Montoya, 2001). Thus, MN scoring during interphase provides a measure of genotoxicity both in the field and also specifically through genotoxic compound exposure in the laboratory as a result of clastogens and/or aneugens (Heddle *et al.*, 1991; Al-Sabti and Metcalfe, 1995). In addition, there are direct indications that MN may also be formed via a nuclear budding mechanism in the interphase of cell division. The formation of such MN reflects a reduced capacity of the organism to expel damaged, amplified, failed replicated or improperly condensed DNA, chromosome fragments without telomeres, and centromeres from the nucleus (Lindberg *et al.*, 2007).

The MN assay involves the scoring of cells that contain one or more MN in the cytoplasm (Schmid, 1975). The assay was first developed as a routine *in vivo* mutagenicity assay for detecting chromosomal mutations in mammalian studies (Boller and Schmid, 1970; Heddle, 1973). Hooftman and Raat (1982) were the first to successfully apply the assay to aquatic species when they demonstrated the induction of MN in erythrocytes of the eastern mudminnow (*Umbra pygmaea*) following waterborne exposure to the known mutagen ethyl methanosulfate (EMS). Following these initial experiments, other studies have validated the detection of MN as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review, see Chaudhary *et al.*, 2006; Udroiu, 2006; Bolognesi and Hayashi, 2011). In fish, most studies have used circulating erythrocytes (blood) cells, but samples from a number of tissues, such as liver, kidney, gill, or fin epithelium, can also be used (Arkhipchuk and Garanko, 2005; Baršienė *et al.*, 2005b, 2006a; Rybakovas *et al.*, 2009).

Environmental genotoxicity levels in organisms from the North Sea, Mediterranean, and northern Atlantic have been described in indigenous fish and mussel species inhabiting reference and contaminated sites (Wrisberg *et al.*, 1992; Bresler *et al.*, 1999; Baršienė *et al.*, 2004, 2008a, 2010a; Bagni *et al.*, 2005; Bolognesi *et al.*, 2006a; Magni *et al.*, 2006; Fernández *et al.*, 2011). Concerns about environmental genotoxicity in oil and gas industrial areas of the North Sea were raised when comparatively high levels of MN incidences were detected in mussels (*Mytilus edulis*) and Atlantic cod (*Gadus morhua*) caged close to the oil platforms (Hylland *et al*., 2008). Increased environmental genotoxicity and cytotoxicity have been described in an offshore Ekofisk oil extraction field (Rybakovas *et al*., 2009). The Water Column Monitoring Programme indicated increased genotoxicity in caged mussels in sites that were close to the Ekofisk oil platform, indicating the ability to pinpoint source discharges with genotoxic endpoints in caged mussels (J. Baršienė, pers. comm.; Brooks *et al*., 2011). Significant MN elevation in fish and mussels was found after exposure to the crude oil extracted from the North Sea (Baršienė *et al*., 2006a; Bolognesi *et al*., 2006a; Baršienė and Andreikėnaitė, 2007; Andreikėnaitė, 2010) and from Arctic zones (J. Baršienė, pers. comm.).

The frequency of the observed MN may be considered to be a suitable index of accumulated genetic damage during the cell lifespan, providing a time-integrated response of an organism's exposure to contaminant mixtures is available. Depending on the lifespan of each cell type and on their mitotic rate in a particular tissue, the MN frequency may provide early warning signs of cumulative stress (Bolognesi and Hayashi, 2011). Caged mussels in Genoa Harbour, which is heavily polluted by aromatic hydrocarbons, showed a continuous increase in MN in mussel gill cells, reaching a plateau after a month of caging (Bolognesi *et al*., 2004). After 30 days of caging in the Cecina estuary on the Tyrrhenian coast, mussels showed a twofold increase of MN incidence in gill cells (Nigro *et al*., 2006). A gradient-related increase in MN was found in haemocytes of mussels and liver erythrocytes of Atlantic cod caged for 5–6 months at Norwegian oil platforms in the North Sea (Hylland *et al*., 2008; Brooks *et al*., 2011). Furthermore, recovery was detected in the sinking zone of the oil tanker MT "Haven" using the MN test in caged mussels 10 years after the oil spill (Bolognesi *et al*., 2006a). In this respect, an increase in MN frequency represents a time-integrated response to cumulative stress.

10.2 Short description of the methodology

10.2.1 Target species

The MN frequency test has generally been applied to organisms where other biological effects, techniques, and contaminant levels are well documented. That is the case for mussels and for certain demersal fish species (such as European flounder, dab, Atlantic cod, or red mullet), which are routinely used in biomonitoring programmes and to assess contamination in western European marine waters (see Figures 10.1 and 10.2; Table 10.1). However, the MN assay can also be adapted for alternative sentinel species using site-specific monitoring criteria.

When selecting an indicator fish species, consideration must be given to its karyotype, as many teleosts are characterized by an elevated number of small chromosomes (Udroiu, 2006). This means that in certain cases MN formed after exposure to clastogenic contaminants will be very small and hard to detect by light microscopy. This can be addressed to a certain extent by using fluorescent staining. After selecting target/suitable species, researchers should also ensure that other factors including age, sex, temperature, and diet are similar between the sample groups. If conducting transplantation studies, consideration needs to be given to the cell turnover rate of the tissue being examined to ensure sufficient cells have gone through cell division. For example, if using blood, the regularities of erythropoiesis should be known prior to sampling.

In general, indigenous, ecologically and economically important fish and mollusc species could serve as indicator species for biomonitoring of environmental genotoxicity levels, for screening of genotoxins distribution, or for assessments of genotoxicity effects from contaminant spills or effluent discharges. For monitoring in deep waters in northern latitudes (deeper than 1000 m), Arctic rockling (*Onogadus argentatus*) and amphipods (*Eurythenes gryllus*) are suitable species. In equatorial regions of the Atlantic, the indicator fish species *Brachydeuterus auritus, Cynoglossus senegalensis,* and *Cynoponticus ferox* are available for MN analysis (J. Baršienė, pers. comm.).

10.2.2 Target tissues

The majority of studies to date have used haemolymph and gill cells of molluscs and peripheral blood cells of fish for MN analysis (Bolognesi and Hayashi, 2011). There are other studies (albeit limited) available describing the use of blood cells of fish from other tissues, such as liver, kidney, and gills (Baršienė *et al*., 2006b; Rybakovas *et al*., 2009), and also other cells (e.g. fin cells; Arkhipchuk and Garanko, 2005) The application of the MN assay to blood samples of fish is particularly attractive as the method is non-destructive, easy to undertake, and results in an easily quantifiable number of cells present on the blood smears for microscopic analysis. However, studies must be undertaken to assess the suitability of any species or cell type analysed. For example, it is known that Atlantic cod have very low levels of MN in blood erythrocytes in specimens from reference sites or control groups in laboratory exposures to crude oil. Furthermore, it has been shown that MN induction in cod blood erythrocytes and erythrocytes from different haematopoietic tissues (liver, kidney, gill, and spleen) differ significantly after 3 wk of exposure to Stafjord B crude oil. In multiple laboratory exposures (108 exposure groups of cod), developing liver and kidney erythrocytes proved to be the most sensitive endpoint and most suitable approach for the assessment of oil pollution in the northern Atlantic and North Sea (Baršienė *et al*., 2005a, 2006b). Liver can also be used as a target organ in *in situ* exposures with turbot and halibut (caged or laboratory; J. Baršienė, pers. comm.).

10.2.3 Sample and cell scoring size

The detected MN frequency in fish erythrocytes is approximately six- to tenfold lower than in mussels and clams. The large interindividual variability associated with the low baseline frequency for this biomarker confirms the need for the scoring of a consistent number of cells in an adequate number of animals for each study point. Sample size in most studies conducted with mollusc species has been 1000–2000 cells scored per animal (Bolognesi *et al*., 1996, 2004, 2006b; Izquierdo *et al*., 2003; Hagger *et al*., 2005; Koukouzika and Dimitriadis, 2005, 2008; Magni *et al*., 2006; Baršienė *et al*., 2006b,c, 2008b, 2010a,b; Kopecka *et al*., 2006; Nigro *et al*., 2006; Schiedek *et al*., 2006; Francioni *et al*., 2007; Siu *et al*., 2008), and previous reviews have suggested that when using fish erythrocytes, at least 2000–4000 cells should be scored per animal (Bolognesi *et al*., 2006a; Udroiu, 2006). Previously, scorings of 5000–10 000 fish erythrocytes were used for MN analysis (Baršienė *et al*., 2004). Since 2009–2010, the frequency of MN in fish from the North and Baltic Seas was mostly scored in 4000 cells. In stressful, heavily polluted zones, the scoring of 5000–10 000 cells in fish is still recommended.

In mussels, sample size in MN assays ranges from 5 to 20 mussels per site, as reported in the literature (Venier and Zampieron, 2005; Baršienė *et al*., 2004, 2008a,b; Bolognesi *et al*., 2004; Baršienė and Rybakovas, 2006; Francioni *et al*., 2007; Siu *et al*., 2008). Evidence suggests that a sample size of 10 specimens per site is enough for the assessment of environmental genotoxicity levels and evaluation of the existence of genetic risk zones. In heavily polluted sites, MN analysis in 15–20 specimens is recommended, because of the higher individual variation of the MN frequency. MN analysis in more than 20 mussel or fish specimens shows only a minor change of the MN means (figure 1 in Fang *et al.*, 2009; J. Baršienė, pers. comm.).

10.2.4 MN identification criteria

Most of the studies have been performed using diagnostic criteria for MN identification developed by several authors (Heddle, 1973; Heddle *et al.*, 1991; Carrasco *et al.*, 1990; Al-Sabti and Metcalfe, 1995; Fenech, 2000; Fenech *et al.*, 2003):

- The size of MN is smaller than 1/3 of the main nucleus.
- MN are round- or ovoid-shaped, non-refractive chromatin bodies located in the cytoplasm of the cell and can, therefore, be distinguished from artefacts such as staining particles.
- MN are not connected to the main nuclei, and the micronuclear boundary should be distinguishable from the nuclear boundary.

After sampling and cell smear preparation, slides should be coded. To minimize technical variation, the blind scoring of MN should be performed without knowledge of the origin of the samples. Only cells with intact cellular and nuclear membranes can be scored. Particles with colour intensity higher than that of the main nuclei were not counted as MN. The area to be scored should first be examined under low magnification to select the part of the slide showing the best quality (good staining, non-overlapping cells). Scoring of micronuclei should then be undertaken at 1000× magnification.

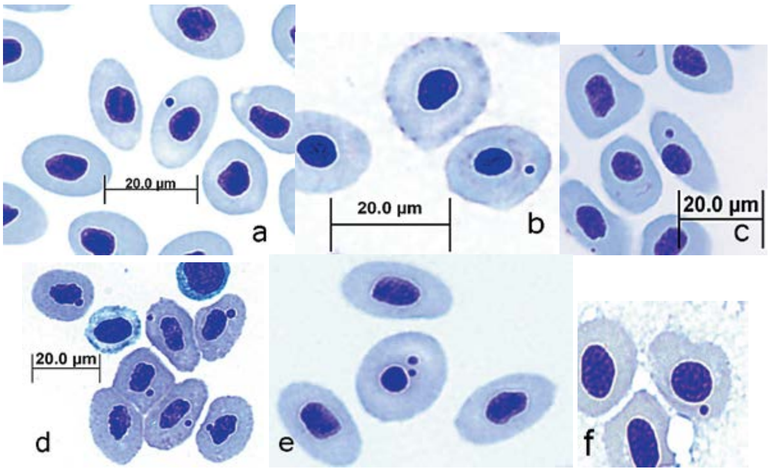


Figure 10.1. Micronuclei in blood erythrocytes of (a) *Platichthys flesus*, (b) *Limanda limanda*, (c) *Zoarces viviparus*, (d) *Clupea harengus*, (e) two MN in *Limanda limanda*, and (f) MN liver erythrocytes of *Gadus morhua*. Images from NRC (Nature Research Center) database.

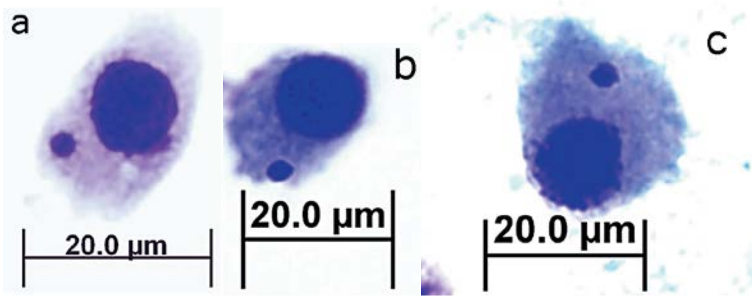


Figure 10.2. Micronuclei in gill cells of (a) *Mytilus edulis*, (b) *Macoma balthica*, and in (c) haemocytes of *Chlamys islandica*. Images from NRC database.

10.3 Confounding factors

Earlier studies on MN formation in mussels have disclosed a significant influence of environmental and physiological factors (Dixon *et al.*, 2002). Therefore, the role of the confounding factors should be considered prior to the application of MN assay in biomonitoring programmes, as well as in descriptions of genetic risk zones or ecosystem health assessments.

10.3.1 Water temperature

MN induction is a cell cycle-related process and depends on water temperature, which is a confounding factor for the mitotic activity in poikilotherm animals. Several studies have demonstrated that baseline frequencies of MN in mussels are related to water temperature (Brunetti *et al.*, 1988, 1992; Kopecka *et al.*, 2006). Baseline frequencies of MN are regarded as the incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). In fish, MN frequencies also showed seasonal differences in relation to water temperature, with lower MN levels in winter than in autumn (Rybakovas *et al.*, 2009). This was assumed to be an effect of higher mitotic activity and MN formation in response to high water temperatures in autumn (Brunetti *et al.*, 1988). In addition, it has been reported that increases in water temperature (4–37°C) can increase the ability of genotoxic compounds to damage DNA (Buschini *et al.*, 2003).

10.3.2 Types of cells

MN may be seen in any type of cell, both somatic and germ, and thus the MN test can be carried out in any active tissue. Nevertheless, there are some limitations to using different types of cells, for example, agranular and granular haemocytes in mussels. There are also differences between MN induction levels in mussel haemolymph and gill cells, mainly because gills are primary targets for the action of contaminants. The anatomical architecture of the spleen in fish does not allow erythrocyte removal in the spleen (Udroiu, 2006) as it does in mammals.

10.3.3 Salinity

The influence of salinity on the formation of MN was observed in mussels from the Danish coast located in the transitional zone between the Baltic and North Seas. No relationship between salinity and MN frequencies in mussels could be found for mussels from the North Sea (Karmsund zone), Wismar Bay, and Lithuanian coast. Similar results were found for *Macoma balthica* from the Baltic Sea—from the gulfs of Bothnia, Finland, Riga, and Lithuanian EEZ (J. Baršienė, pers. comm.).

10.3.4 Size

Because linear regression analysis of animal length and MN induction shows that size could be a confounding factor, sampling of organisms of similar size should take place (J. Baršienė, pers. comm.). It should also be noted that size is not always indicative of age; therefore, age could also potentially affect the genotoxicity response in fish.

10.3.5 Diet

Results have shown that MN formation was not influenced in mussels maintained under simple laboratory conditions without feeding (Baršienė and Rybakovas, 2006).

10.4 Ecological relevance

Markers of genotoxic effects reflect damage to genetic material of organisms and thus receive a lot of attention (Moore *et al.*, 2004a). Different methods have been developed for the detection of both double- and single-strand breaks of DNA, DNA adducts, MN formation, and chromosome aberrations. The assessment of chemical-induced genetic damage has been widely utilized to predict the genotoxic, mutagenic, and carcinogenic potency of a range of substances; however, these investigations have mainly been restricted to humans or mammals (Siu *et al.*, 2004). MN formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is, however, limited knowledge of the relationship between MN formation and effects on offspring in aquatic organisms. With growing concern over the presence of genotoxins in the sea, the application of cytogenetic assays to ecologically relevant species offers the chance to perform early tests on health in relation to exposure to contaminants.

10.5 Applicability across the OSPAR maritime area

Large-scale and long-term studies took place from 2001 to 2010 at the Nature Research Center (NRC, Lithuania) on MN and other abnormal nuclear formations in various fish and bivalve species inhabiting sites in the North Sea, Baltic Sea, Atlantic Ocean, and Barents Sea. These studies revealed the relevance of environmental genotoxicity levels in ecosystem assessments. The NRC established a large database on MN and other nuclear abnormalities in 13 fish species from the North Sea, Barents

Sea, and Atlantic Ocean, and in eight fish species and in mussels, scallops, and clams (*Macoma balthica*) from the Baltic Sea. Fish and bivalve species were collected from 85 sites in the North Sea and Atlantic and from 117 coastal and offshore sites in the Baltic (Figures 10.3 and 10.4). Monitoring of MN and other nuclear abnormalities levels was performed (2–8 times) in many sites of the North and Baltic Seas. Data on MN levels in organisms inhabiting deep-sea and Arctic zones are also available (Table 10.1).

The validation of the MN assay was done with indigenous and cultured mussels (*M. edulis*), Atlantic cod, turbot, halibut, and long rough dab in multiple laboratory exposures to crude oil from the North Sea and Barents Sea, to produced-water discharges from the oil platforms, and to other contaminants. Additional active monitoring using mussels and Atlantic cod took place in the Ekofisk, Statfjord, Troll oil platform, oil refinery zones, some northern Atlantic sites, and in sites heavily polluted by copper or polycyclic aromatic hydrocarbons.

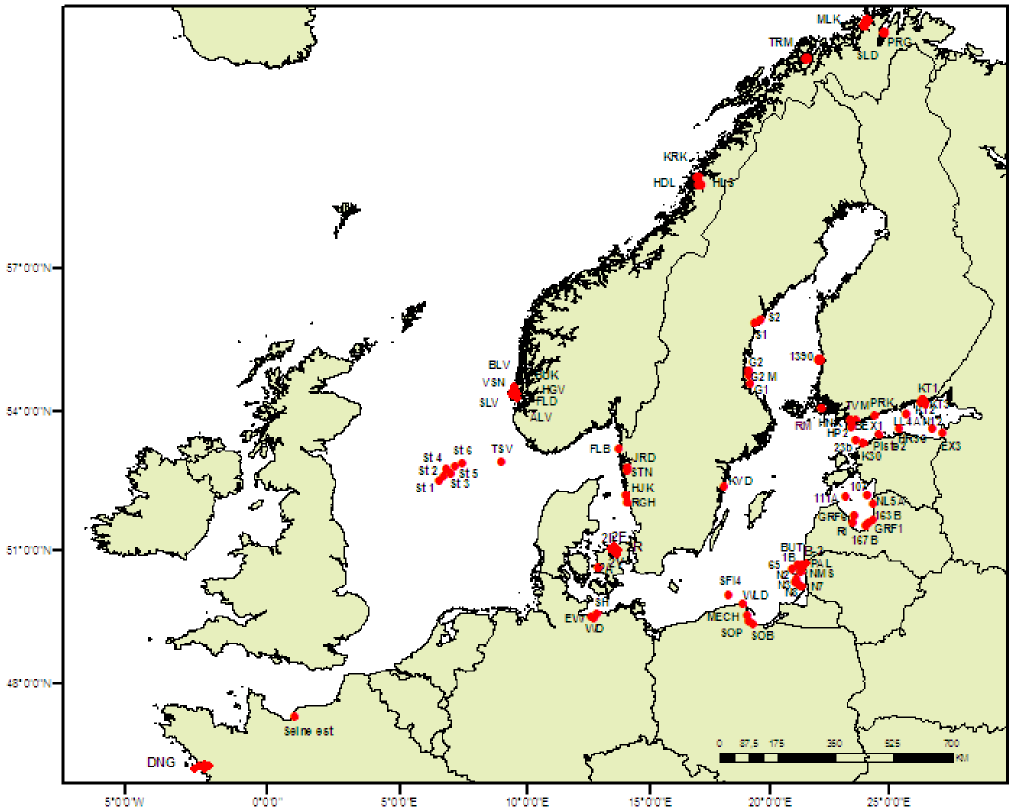


Figure 10.3. Sampling stations of bivalve molluscs for the micronuclei studies (NRC, Lithuania).

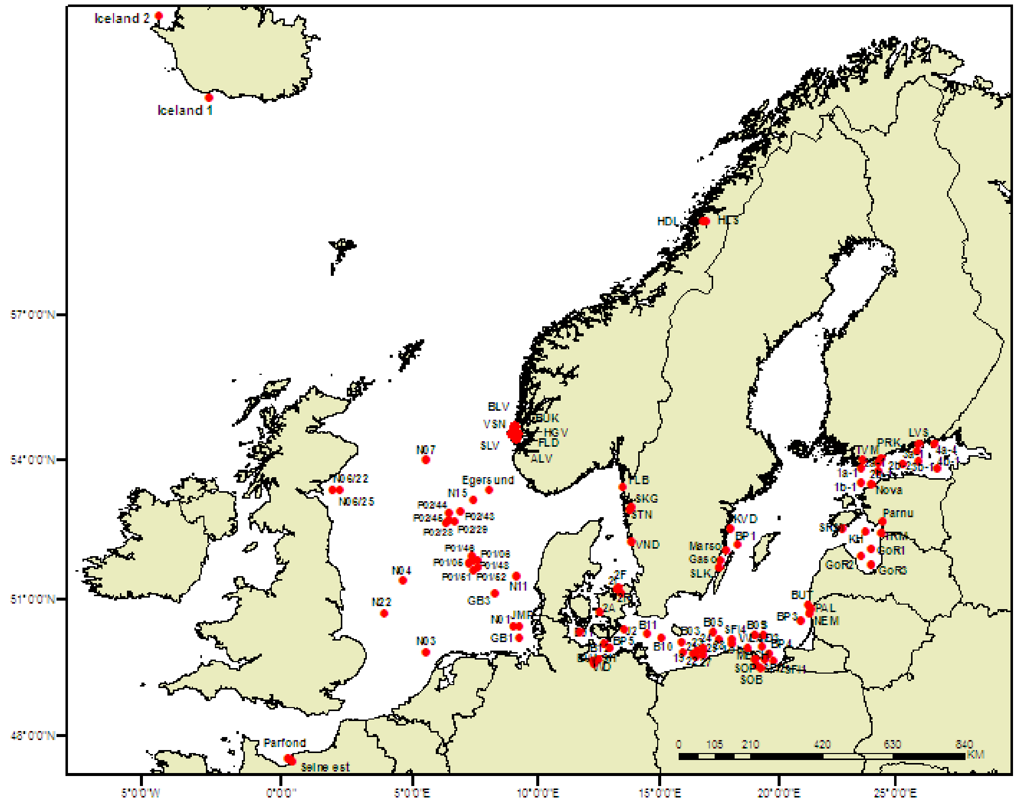


Figure 10.4. Sampling stations of fish species used for the micronuclei studies (NRC, Lithuania).

10.6 Background responses

Baseline or background frequency of MN can be defined as the incidence of MN observed in the absence of environmental risk or before exposure to genotoxins (Fenech, 1993). As mentioned above, several studies have demonstrated that MN baseline frequencies depend on water temperature. In fish, MN frequencies lower than 0.05‰ (the Baltic Sea) and lower than 0.1‰ (the North Sea) have been suggested by Rybakovas *et al.* (2009) as a reference level in the peripheral blood erythrocytes of the flatfish flounder (*Platichthys flesus*), dab (*Limanda limanda*), and also cod (*Gadus morhua*) after analysing fish from 12 offshore sites in the Baltic Sea (479 specimens) and 11 sites in the North Sea (291 specimens). For unpolluted sites in the Mediterranean Sea, baseline MN levels in gills of *M. galloprovincialis* have been set depending on water temperature to 1% at temperatures below 15°C, 2% between 15 and 20°C, and 3% above 20°C (Brunetti *et al.*, 1992).

The frequencies of MN in marine species sampled from field reference sites are summarized in Table 10.1. In addition, the frequencies of MN in blood erythrocytes of fish and in gill cells of mussels taken to uncontaminated sites are shown in Table 10.2).

Table 10.1. Reference levels of micronuclei (MN 1000^{-1} cells) in European marine species *in situ*

Species	Tissue	Location	Response MN 1000^{-1} cells	Reference
Mytilus galloprovincialis	Gills	Adriatic and Tyrrhenian Sea	1.0 at 15°C 2.0 at 15–20°C 3.0 at >20°C	Brunetti *et al.* (1992)
M. galloprovincialis	Haemolymph	Mediterranean coast	4.2 ± 0.7	Burgeot *et al.* (1996b)

M. galloprovincialis	Gills	La Spiezia Gulf, Ligurian Sea	3.0 ± 2.0	Bolognesi *et al.* (1996)
M. galloprovincialis	Gills, Haemolymph	Venice Lagoon	0.73–1.42	Dolcetti and Venier (2002)
M. galloprovincialis	Haemolymph	Strymonikos Gulf, Mediterranean Sea	0.30; 1.30	Dailianis *et al.* (2003)
M. edulis	Gills	Gijon coast, Spain	1.42	Izquierdo *et al.* (2003)
M. galloprovincialis	Gills	Strymonikos Gulf, Mediterranean Sea	1.30	Dailianis *et al.* (2003)
M. galloprovincialis	Haemolymph	Venice lagoon	0.44	Pampanin *et al.* (2005)
M. galloprovincialis	Gills	Tyrrhenian Sea	5.4	Nigro *et al.* (2006)
M. galloprovincialis	Gills	Gulf of Oristano, Mediterranean Sea	2.94–4.70	Magni *et al*. (2006)
M. galloprovincialis	Haemolymph	Adriatic Sea	1.0–1.5	Klobučar *et al.* (2008)
M. galloprovincialis	Haemolymph	Adriatic Sea	1.38–1.75	Pavlica *et al.* (2008)
M. galloprovincialis	Gills	Gulf of Patras	≈2.0	Pytharopoulou *et al.* (2008)
M. galloprovincialis	Gills	Algerian coast	0.0–1.18	Taleb *et al.* (2009)
M. galloprovincialis	Haemolymph	Algerian coast	1.6–2.47	Taleb *et al.* (2009)
M. galloprovincialis	Gills	Western Mediterranean	1.9–2.1	Fernández *et al.* (2011)
M. edulis	Haemolymph	Langesundfjord (Norway, rock)	0.90	Wrisberg *et al.* (1992)
M. edulis	Haemolymph	Store Belt (Denmark)	0.89	Wrisberg *et al.* (1992)
M. edulis	Gills	North Sea (Norwegian coast and Karmsund fjords)	1.05 ± 0.32	Baršienë *et al.* (2004)
M. edulis	Gills	North Sea (Göteborg coast)	0.71 ± 0.12	Baršienė *et al.* (2008a)
M. edulis	Haemolymph	North Sea	1.24 ± 0.37	Brooks *et al.* (2011)
M. edulis	Gills	Baltic Sea	0.37 ± 0.09	Baršienė *et al.* (2006c)
M. trossulus	Gills	Baltic Sea	2.07 ± 0.32	Baršienė *et al.* (2006c); Kopecka *et al.* (2006)
Macoma balthica	Gills	Baltic Sea	0.53–1.28	Baršienė *et al.* (2008b); J. Baršienė (pers. comm.)
M. balthica	Gills	Stockholm archipelago	0.4	Smolarz and Berger (2009)
Limanda limanda	Blood, kidney erythrocytes	North Sea	0.02 ± 0.01	Rybakovas *et al.* (2009)
Platychthys flesus	Blood erythrocytes	Atlantic Ocean	0.06 ± 0.04	J. Baršienė (pers. comm.)
P. flesus	Blood erythrocytes	North Sea	0.04 ± 0.03	Baršienė *et al.* (2008a)
P. flesus	Blood erythrocytes	Baltic Sea	0.15 ± 0.03	Baršienė *et al.* (2004)
P. flesus	Blood erythrocytes	Baltic Sea	0.0 ± 0.0	Köhler and Ellesat (2008)
P. flesus	Blood erythrocytes	Baltic Sea	0.08 ± 0.02	Napierska *et al.* (2009)
P. flesus	Blood erythrocytes	UK estuaries	0.27–0.66	B. P. Lyons (pers. comm.)
Zoarces viviparus	Blood erythrocytes	Baltic Sea	0.02 ± 0.02	J. Baršienė (pers. comm.)
Gadus morhua	Blood, kidney erythrocytes	North Sea	0.03 ± 0.02	Rybakovas *et al.* (2009)
G. morhua	Blood, kidney erythrocytes	Baltic Sea	0.03 ± 0.02	Rybakovas *et al.* (2009)
Clupea harengus	Blood erythrocytes	Baltic Sea	0.03 ± 0.03	J. Baršienė (pers. comm.)
Symphodus melops	Blood erythrocytes	North Sea	0.08 ± 0.04	Baršienė *et al.* (2004)
Scophthalmus maximus	Blood erythrocytes	Baltic Sea	0.10 ± 0.04	J. Baršienė (pers. comm.)
Perca fluviatilis	Blood erythrocytes	Baltic Sea	0.06 ± 0.02	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Mugil cephalus	Blood erythrocytes	Mediterranean coast, Turkey	0.82–2.07	Çavaş and Ergene-Gözükara (2005)
M. cephalus	Gill cells	Mediterranean coast, Turkey	1.84–2.91	Çavaş and Ergene-Gözükara (2005)
Mullus barbatus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.33[a]	Bolognesi *et al.* (2006b)
Dicentrarchus labrax	Blood erythrocytes	La Spiezia Gulf (Italy)	0.75[a]	Bolognesi *et al.* (2006b)

Pagellus mormyrus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.4[a]	Bolognesi *et al.* (2006b)
Sargus sargus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Seriola dumerili	Blood erythrocytes	La Spiezia Gulf (Italy)	0.38[a]	Bolognesi *et al.* (2006b)
Serranus cabrilla	Blood erythrocytes	La Spiezia Gulf (Italy)	0.0[a]	Bolognesi *et al.* (2006b)
Sparus auratus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.12[a]	Bolognesi *et al.* (2006b)
Sphyraena sphyraena	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Trachurus trachurus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25[a]	Bolognesi *et al.* (2006b)
Mugil cephalus	Blood erythrocytes	Mediterranean Goksu Delte, Turkey	1.26 ± 0.40	Ergene *et al.* (2007)
Mullus barbatus	Blood erythrocytes	Western Mediterranean, Spain	0.10–0.16	Martínez-Gómez (2010)
Dicentrarchus labrax	Blood erythrocytes	Eastern Adriatic Sea	1.25 ± 1.97	Strunjak-Perovic *et al.* (2009)

[a]**Number of MN 1000^{-1} studied erythrocytes.**

Note: It is important to ensure that the data are normally distributed (e.g. Kolmogorov–Smirnov test) if the standard deviation is to be used to calculate MN frequency percentiles of the distribution, as this assumes that the data are normally distributed, which may not be the case.

Table 10.2. The reference levels of micronuclei (MN 1000^{-1} cells) in European marine organisms after caging in uncontaminated/reference sites *in situ*

Species	Tissue	Location/exposure time	Response MN 1000^{-1} cells	Reference
Mytilus galloprovincialis	Gills	Ligurian coast/30 days	1.78 ± 1.04[a]	Bolognesi *et al.* (2004)
M. galloprovincialis	Gills	Gulf of Patras/1 month	2.3–2.5	Kalpaxis *et al.* (2004)
M. galloprovincialis	Gills	Haven oil spill area/30 days	3.7 ± 1.62[a]	Bolognesi *et al.* (2006a)
M. galloprovincialis	Gills	Cecina estuary/4 wk	5.4	Nigro *et al.* (2006)
M. galloprovincialis	Haemolymph	Adriatic Sea/1 month	1.0	Gorbi *et al.* (2008)
M. galloprovincialis	Haemolymph	Tyrrhenian coast/1 month	0.27	Bocchetti *et al.* (2008)
M. galloprovincialis	Haemolymph	Algerian coast/1 month	1.6–2.47	Taleb *et al.* (2009)
M. galloprovincialis	Gills	Algerian coast/1 month	0.0–1.18	Taleb *et al.* (2009)
Mytilus edulis	Gills	Visnes copper site (Norway)/3 wk	1.87 ± 0.43	Baršienė *et al.* (2006d)
M. edulis	Gills	Karmsund (Norway)/4 weeks	1.40 ± 0.29	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	North Sea, oil platforms (Norway)/6 wk	2.13 ± 0.48	Hylland *et al.* (2008)
M. edulis	Haemolymph	Seiland site (Norway)/5.5 months	2.60 ± 0.21	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Ekofisk oil platform, North Sea/6 wk	1.24 ± 0.37 (2006) 3.34 ± 0.28 (2008) 2.78 ± 0.50 (2009)	Brooks *et al.* (2011)
M. edulis	Haemolymph	Oil refinery (France, 2004)	3.20 ± 0.36	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Oil refinery (France, 2006)	2.34 ± 0.37	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Oil refinery (Mongstad, 2007)/100 days	2.90 ± 0.40	J. Baršienė (pers. comm.)
M. edulis	Haemolymph	Sea Empress clean reference area (90 days)	0.75 ± 0.46	Lyons (1998)
M. edulis	Haemolymph	Sea Empress clean reference area (110 days)	0.81 ± 0.36	Lyons (1998)
Crassostrea gigas	Gills	MT “Haven” oil spill	1.49 ± 0.79[a]	Bolognesi *et al.*

		area/30 days		(2006a)
Gadus morhua	Liver erythrocytes	North Sea, oil platforms (Norway)/5 wk	0.12 ± 0.05	Hylland *et al.* (2008)
G. morhua	Liver erythrocytes	North Sea, oil platforms (Norway)/6 wk	0.27 ± 0.13	J. Baršienė (pers. comm.)
Boops boops	Erythrocytes	Haven oil spill area/30 days	0.6 ± 0.7[a]	Bolognesi *et al.* (2006b)
Mullus barbatus	Erythrocytes	Haven oil spill area/30 days	0.7 ± 0.6[a]	Bolognesi *et al.* (2006b)
Uranoscopus scaber	Erythrocytes	Haven oil spill area/30 days	1.1 ± 0.5[a]	Bolognesi *et al.* (2006b)

[a]Number of MN per 1000 studied cells

In addition, the range of variation of the frequency of MN in blood erythrocytes of fish and gill cells of *M. galloprovincialis* is displayed in Table 10.3.

Table 10.3. The range of MN frequency fish (blood, liver, kidney erythrocytes), in mussels, clams, scallops, and amphipods (haemolymph, gill, and mantle cells) from different sites in the Atlantic Ocean, North Sea, Baltic Sea, and Mediterranean Sea

Species	Number of sites studied	Tissue	MN frequency range (‰)	Reference
Mytilus edulis	3	Haemolymph	0.89–2.87	Wrisberg *et al.* (1992)
M. edulis	2	Haemolymph	0.90–2.32	Wrisberg *et al.* (1992)
M. edulis	3	Mantle	≈3–7[a]	Bresler *et al.* (1999)
M. edulis	60	Gills, haemolymph	0.37–7.20	Baršienė *et al.* (2004, 2006c, 2008b, 2010a); Baršienė and Rybakovas (2006), Schiedek *et al.* (2006)
Mytilus trossulus	5	Gills	2.07–6.70	Baršienė *et al.* (2006c); Kopecka *et al.* (2006)
M. galloprovincialis	13	Gills	1.8–24	Brunetti *et al.* (1988); Scarpato *et al.* (1990); Bolognesi *et al.* (2004); Nigro *et al.* (2006)
M. galloprovincialis	3	Gills	2–12	Kalpaxis *et al.* (2004)
M. galloprovincialis	5	Haemolymph	1.38–6.50	Pavlica *et al.* (2008)
M. galloprovincialis	3	Gills	1.2–11.8	Taleb *et al.* (2009)
M. galloprovincialis		Gills	0–22	Fernández *et al.* (2011)
Macoma balthica	29	Gills	0.53–11.23	Baršienė *et al.* (2008b); J. Baršienė (pers. comm.)
Chlamys islandica	3	Haemolymph	3.50–5.83	J. Baršienė (pers. comm.)
Eurythenes gryllus	2	Haemolymph	0.35–0.52	J. Baršienė (pers. comm.)
Limanda limanda	3	Blood	≈2–5[b]	Bresler *et al.* (1999)
L. limanda	26	Blood, kidney	0.02–1.22	Rybakovas *et al.* (2009); J. Baršienė (pers. comm.)
Platychthys flesus	3	Blood	≈ 2–6[b]	Bresler *et al.* (1999)
P. flesus	53	Blood, kidney	0.08–1.45	Baršienė *et al.* (2004, 2005a, 2008a); Napierska *et al.* (2009); J. Baršienė (pers. comm.)
Zoarces viviparus	40	Blood	0.02–0.81	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Gadus morhua	19	Liver, blood,	0.0–0.64	Rybakovas *et al.* (2009); Baršienė *et al.* (2010a)
Symphodus melops	9	Blood	0.07–0.65	Baršienė *et al.* (2004, 2008a)
Clupea harengus	32	Blood	0.03–0.92	J. Baršienė (pers. comm.)
Melanogrammus aeglefinus	3	Liver	0.06–0.75	J. Baršienė (pers. comm.)

Scophthalmus maximus	4	Blood, liver, kidney	0.10–0.93	J. Baršienė (pers. comm.)
Perca fluviatilis	14	Blood	0.06–1.15	Baršienė *et al.* (2005a); J. Baršienė (pers. comm.)
Brachydeuterus auritus	3	Liver	0.28–0.85	J. Baršienė (pers. comm.)
Cynoglossus senegalensis	2	Liver	0.33–0.45	J. Baršienė (pers. comm.)
Cynoponticus ferox	2	Liver	0.13–0.96	J. Baršienė (pers. comm.)
Rhinobatos irvinei	1	Liver	0.50	J. Baršienė (pers. comm.)
Onogadus argentatus	2	Liver	0.23–0.47	J. Baršienė (pers. comm.)

[a]Frequency of MN in cells. [b]Frequency of MN in erythrocytes

10.7 Assessment criteria

Assessment criteria (AC) have been established by using data available from studies for molluscs and fish in the North Sea, northern Atlantic (NRC database), and Mediterranean area (Table 10.4). The background/threshold level of MN incidence is calculated as the empirical 90% percentile (P90). Until more data become available, values should be interpreted from existing national datasets. It should be noted that these values are provisional and require further validation as data become available from the ICES database.

The 90th percentile (P90) separates the upper 10% of all values in the group from the lower 90%. The rationale for this decision was that elevated MN frequency would lie above P90, whereas the majority of values below P90 belong to unexposed, weakly–medium exposed or non-responding adapted individuals. P90 values were calculated for those stations/areas which were considered reference stations (i.e. no known local sources of contamination or those areas that were not considered unequivocally as reference sites but were less influenced by human and industrial activity).

ACs in bivalves (*Mytilus edulis*, *Mytilus trossulus*, *Macoma balthica*, and *Chlamys islandica*; data from MN analysis in 4371 specimens) and in fish (*Limanda limanda*, *Zoarces viviparus*, *Platichthys flesus*, *Symphodus melops*, *Gadus morhua*, *Clupea harengus*, and *Melogrammus aeglefinus*; data from MN analysis in 4659 specimens) from the North Sea, Baltic Sea, and northern Atlantic have been calculated using NRC (Lithuania) databases with data from five or more reference locations (Table 10.1).

ACs for mussels (*Mytilus galloprovincialis*) and red mullet (*Mullus barbatus*) have been estimated using available data from the Spanish Institute of Oceanography (IEO, Spain). This dataset was obtained using *M. galloprovincialis* from reference stations along the northern Iberian shelf in spring 2003, namely Cadaqués and Medas Islands. In the case of red mullet, background values were derived from the results obtained in Almeria and Málaga areas (southeast Spain). Because significant sexual differences were not observed in red mullet, data of both genders were considered.

Table 10.4. Assessment criteria of MN frequency levels in different bivalve mollusc and fish species

SPECIES	SIZE (cm)	TEMPERATURE (°C)	REGIONAL AREA	TISSUE	BR	ER	*n*
Mytilus edulis	3–4	11–17	Atlantic–North Sea	Haemolymph, gills	<2.51	>2.51	1 280
M. edulis	1.5–3	8–18	Baltic Sea	Gills	<2.50	>2.50	1 810
M. edulis caged for 4–6 wk	3–4	7–9	North Sea	Haemolymph	<4.1	>4.1	44
M. edulis caged for 4–6 wk	3–4	9–16	North Sea	Haemolymph	<4.06	> 4.06	656
M. trossulus	2–3	3–15	Baltic Sea	Gills	<4.50	>4.50	230

Macoma balthica	1–3	13–18	Baltic Sea	Gills	<2.90	>2.90	330
M. galloprovincialis	3–4	13	Western Mediterranean	Gills	<3.87	>3.87	12
Chlamys islandica	4–5	2–4	North Sea	Haemolymph	<4.5	>4.5	65
Zoarces viviparous	17–30	15–17	North Sea	Erythrocytes	<0.28	>0.28	226
Zoarces viviparous	15–32	7–17	Baltic Sea	Erythrocytes	<0.38	>0.38	824
Limanda limanda	19–24	8–17	North Sea	Erythrocytes	<0.37	>0.37	544
Limanda limanda	18–25	8–17	Baltic Sea	Erythrocytes	<0.49	>0.49	117
Platichthys flesus	20–28	15–17	Atlantic-North Sea	Erythrocytes	<0.33	>0.33	62
Platichthys flesus	17–39	10–17	Baltic Sea coastal	Erythrocytes	<0.29	>0.29	828
Platichthys flesus	18–40	6–18	Baltic Sea offshore	Erythrocytes	<0.23	>0.23	970
Symphodus melops	12–21	13–15	Atlantic-North Sea	Erythrocytes	<0.36	>0.36	158
Gadus morhua	20–48	13–15	Atlantic-North Sea	Erythrocytes	<0.38	>0.38	340
Gadus morhua	20–48	13–15	Baltic Sea	Erythrocytes	<0.38	>0.38	50
Clupea harengus	19–25	5–10	Atlantic-North Sea	Erythrocytes	<0.32	>0.32	60
Clupea harengus	16–29	6–18	Baltic Sea	Erythrocytes	<0.39	>0.39	450
Melogrammus aeglefinus	27–44	8–14	North Sea	Erythrocytes	<0.30	>0.30	30
Mullus barbatus	12–18	17	Western Mediterranean	Erythrocytes	<0.32	>0.32	64

BR, background response; ER, elevated response; *n*, number of specimens analysed.

10.8 Quality assurance

The MN test was found to be a useful *in vivo* assay for genotoxicity testing. However, many aspects of its protocol need to be refined, knowledge of confounding factors should be improved, and interspecies differences need further investigation. In 2009, an interlaboratory comparison exercise was organized within the framework of the MED POL programme using *M. galloprovincialis*..

Intercalibration of MN analysis in fish was done between experts from NRC and Caspian Akvamiljo laboratories, as well as between NRC experts and the University of Aveiro, Portugal (Santos *et al*., 2010). It is recommended that these relatively simple interlaboratory collaborations should be expanded to include material from all of the commonly used bioindicator species in 2011/2012.

10.9 Scientific potential

MN analysis in different marine and freshwater species of bivalves and fish is carried out in many European laboratories in Italy, Portugal, Spain, Turkey, Lithuania, the UK, Greece, Germany, Poland, Croatia, Estonia, Russia, Norway, and Ukraine. There are single laboratories in Hungary, Algeria, and Egypt. Highly qualified expert groups are working in Italy, Lithuania, Spain, Turkey, Portugal, and the UK and are able to perform analysis in both invertebrates and vertebrates.

11 Background document: externally visible fish diseases, macroscopic liver neoplasms, and liver histopathology

Thomas Lang, Stephen Feist, Werner Wosniok, and Dick Vethaak

11.1 Summary

Applicability across OSPAR maritime area. Externally visible fish diseases have been used internationally for many years as an integrative response for general biological effects monitoring, measuring the general health status at the individual and population level. The method is used for a variety of fish species, including dab (*Limanda limanda*), flounder (*Platichthys flesus*), and cod (*Gadus morhua*) and is easily adaptable for other species, such as whiting (*Merlangius merlangus*) and haddock (*Melanogrammus aeglefinus*). Methodologies and diagnostic criteria involved in the monitoring of contaminant-specific macroscopic liver neoplasms (= liver nodules) and liver histopathology have largely been developed based on experiences with flatfish species (in Europe, mainly dab and flounder), but can also be adapted to other flatfish species and also to bottom-dwelling roundfish species.

Status of quality assurance. Quality assurance procedures for externally visible fish diseases, macroscopic liver neoplasms, and liver histopathology are in place and operational through ICES activities and under the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) scheme (www.bequalm.org). Largely through activities of the International Council for the Exploration of the Sea (ICES), standardized methodologies for surveys on the occurrence of diseases of flatfish species from the North Sea and adjacent areas have been developed and intercalibrated repeatedly. Practical guidelines have been established for all methodologies involved, including sampling of fish, diagnosis of diseases, reporting of data to ICES, and statistical data analysis. As part of the work carried out in BEQUALM, these guidelines were reviewed and, where necessary, additional details and methodologies for the collection, diagnosis, and reporting of fish disease data are provided. Under BEQUALM, a number of ring-tests and intercalibration workshops were held. *ICES Techniques in Marine Environmental Sciences* (*ICES TIMES*) series publications have been published (nos. 19 and 38).

Influence of environmental variables. Justification is provided that externally visible diseases provide an appropriate indicator of the general health of individuals and populations. The conditions that affect disease are multifactorial and include endogenous and exogenous effects on the immune response of the fish as well as specific and non-specific, contaminant-related effects at differing biological levels of organization. Certain types of non-neoplastic and neoplastic liver lesions (as specified in the guidelines for the Joint Assessment and Monitoring Programme – JAMP/Coordinated Environmental Monitoring Programme – CEMP) are known to be associated with prior exposure to carcinogenic contaminants such as polycyclic aromatic hydrocarbons (PAHs).

Assessment of thresholds. For externally visible diseases, background assessment criteria (BAC) and environmental assessment criteria (EAC) have been defined (see Tables 11.1 and 11.2). Assessment criteria (AC) for non-specific and contaminant-specific liver histopathology and for macroscopic liver neoplasms are under development, and suggestions have been made by the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC, ICES 2009)

and have been further detailed by the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO, ICES 2011, 2012) (see Table 11.2).

Table 11.1. Assessment criteria proposed for the assessment of contaminant-specific effects on fish health in using BAC and EAC (assessment).

Disease category	Background	Elevated response/ above background	Significant response/unacceptable effects
Externally visible diseases (to be used as additional information for the assessment)	FDI level ≤ BAC* (see Table 11.2) *BAC calculated based on the 10th percentile of all standardized sample FDI means	BAC < FDI level ≤ EAC* (see Table 11.2) *EAC calculated based on the mean FDI that is associated with a 10% loss in condition factor	EAC* < FDI level (see Table 11.2) *EAC calculated based on the mean FDI that is associated with a 10% loss in condition factor
Liver histopathology: non-specific (to be used as additional information for the assessment)	Analogous to externally visible diseases	Analogous to externally visible diseases	Analogous to externally visible diseases
Liver histopathology: contaminant-specific	Analogous to externally visible diseases	BAC < FDI level ≤ EAC* *EAC calculated based on the 10th percentile of the standardized FDI sample means for all affected fish (FDI > 0)	EAC* < FDI level *EAC calculated based on the 10th percentile of the standardized FDI sample means for all affected fish (FDI > 0)
Macroscopic liver neoplasms	Analogous to externally visible diseases	Analogous to liver histopathology: contaminant-specific	Analogous to liver histopathology: contaminant-specific

FDI, fish disease index; BAC, background assessment criteria; EAC, environmental assessment criteria.

Table 11.2. Assessment criteria for the assessment of the FDI for externally visible diseases in common dab (*Limanda limanda*) in the North Sea

Sex	Diseases/parasites involved in FDI	Background assessment criteria		Environmental assessment criteria	
		Ungraded diseases	Graded diseases	Ungraded diseases	Graded diseases
F	Ep, Ly, Ul	4.38	1.78	13.9	6.6
M	Ep, Ly, Ul	5.18	2.24	32.8	17.3
F	Ac, Ep, Fi, Hp, Le, Ly, St, Ul, Xc	−6.96	3.07	17.8	7.8
M	Ac, Ep, Fi, Hp, Le, Ly, St, Ul, Xc	10.39	4.59	29.8	13.3
F	Ac, Ep, Hp, Le, Ly, St, Ul, Xc	6.22	2.79	16.0	7.4
M	Ac, Ep, Hp, Le, Ly, St, Ul, Xc	9.51	4.34	26.5	12.4

FDI, fish disease index; Ac, *Acanthochondria cornuta*; Ep, epidermal hyperplasia/papilloma; Fi, acute/healing fin rot/erosion; Hp, hyperpigmentation; Le, *Lepeophtheirus* sp.; Ly, lymphocystis; St, *Stephanostomum baccatum*; Ul, acute/healing skin ulcerations; Xc, X-cell gill disease.

Proposals for assessment tools. The WGPDMO developed a fish disease index (FDI) to be used for the analysis and assessment of fish disease data. BAC and EAC have been agreed upon during the 2011 and 2012 meetings. At the 2009 ICES/OSPAR WKIMC, assessment criteria for macroscopic liver neoplasms and for liver histopathology were proposed.

Final remarks. Some amendments should be made to the JAMP Guidelines for PAH-specific biological effects monitoring related to liver histopathology.

11.2 Assessment of the applicability of fish disease and liver pathology techniques across the OSPAR maritime area

Diseases of wild marine fish have been studied on a regular basis by many ICES Member Countries for more than two decades. Disease surveys are often integrated with other types of biological and chemical investigations as part of national monitoring programmes aiming at an assessment of the health of the marine environment, particularly in relation to the impact of human activities (Lang, 2002).

On an international level, fish disease data have been used for environmental assessments in the framework of the North Sea Task Force and its Quality Status Report (North Sea Task Force, 1993), the OSPAR Quality Status Report 2000 (OSPAR, 2000) and in the 3rd and 4th HELCOM assessments (HELCOM, 1996, 2002). Studies on externally visible diseases, macroscopic liver neoplasms (= liver nodules), and liver histopathology are on the list of techniques for general and contaminant-specific, biological effects monitoring as part of the OSPAR pre-CEMP (see Table 11.4 and Annex 1).

At present, annual or biannual fish disease surveys in the North Sea are carried out by Germany (vTI, Institute of Fisheries Ecology, Cuxhaven), the Netherlands (RIKZ), and the UK (Cefas, Weymouth; Marine Scotland, Aberdeen). However, more data are available from monitoring programmes that were terminated in the 1990s or early 2000s (e.g. carried out by Belgium, Denmark, and Sweden).

The following environmental monitoring programmes incorporating pathology and diseases of marine organisms are routinely performed in the OSPAR area:

- **Germany**: Surveys are carried out twice a year in offshore areas of the North Sea and the southwestern Baltic Sea. The major target fish species in the North Sea is dab (*Limanda limanda*), and flounder (*Platichthys flesus*) and cod (*Gadus morhua*) in the Baltic Sea. Externally visible diseases/parasites and liver anomalies (macroscopic and histopathological) are recorded according to ICES guidelines. The data are submitted to the ICES Data Centre.
- **The Netherlands**: Diseases surveys are done annually in three North Sea offshore areas, sites in the western Wadden Sea and in coastal zone of the Eastern Scheldt, with dab and flounder as target species. Externally visible diseases/parasites and liver anomalies (macroscopic and histopathological) are recorded according to ICES guidelines. The data are submitted to the ICES Data Centre.
- **UK**: The Clean Seas Environment Monitoring Programme (CSEMP) was established to detect long-term trends in physical, biological, and chemical variables at selected estuarine and coastal sites in the North Sea, Irish Sea, and the English Channel, with 25–30 offshore areas included. The biological effect component of this programme includes assessment of the disease status of target flatfish species (dab and flounder). In addition, data on diseases and parasites in commercial species are also collected. Estuarine monitoring activities have been undertaken more recently using flounder and viviparous blenny (*Zoarces viviparus*) as the target species. In Scotland, externally visible diseases/parasites and liver anomalies of dab, cod, and haddock (*Melanogrammus aeglefinus*) are monitored at sampling sites in the Firth of Forth, east of Orkney, and in the Moray Firth. Diseases are recorded according to ICES guidelines, and the data are submitted to the ICES Data Centre via the UK's Marine Environment Monitoring and Assessment National database (MERMAN).

Many of these national programmes have increasingly evolved into integrated monitoring programmes, including studies on chemical contamination and on biological effects of contaminants.

Externally visible disease studies are being conducted in a variety of fish species, including dab (*Limanda limanda*), flounder (*Platichthys flesus*), and cod (*Gadus morhua*), and methodologies are easily adaptable for other species such as whiting (*Merlangius merlangus*) and haddock (*Melanogrammus aeglefinus*). Methodologies and diagnostic criteria involved in the monitoring of contaminant-specific liver neoplasms and liver histopathology have largely been developed based on studies with flatfish species, in Europe, mainly dab and flounder, but can also be adapted to other flatfish species, such as plaice (*Pleuronectes platessa*) or long rough dab (*Hippoglossoides platessoides*), and possibly also to bottom-dwelling roundfish species, such as dragonet species (*Callionymus* spp.) or viviparous blenny (*Zoarces viviparus*).

In conclusion, it can be stated that fish disease and liver histopathology techniques are applicable across the OSPAR maritime area. The application of the fish disease index (FDI) facilitates a comparison of disease data over larger geographical areas and between species (see Section 11.6).

11.3 Status of quality assurance techniques for fish diseases and liver pathology

Since the early 1980s, ICES has played a leading role in the initiation and coordination of fish disease surveys and has contributed considerably to the development of standardized methodologies. Through the work of the ICES Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), its offspring, the Sub-Group/Study Group on Statistical Analysis of Fish Disease Data in Marine Stocks (SGFDDS) (1992–1994), and the ICES Secretariat, quality assurance procedures have been implemented at all stages, from sampling of fish to submission of data to the ICES Data Centre and to data assessment.

A number of ICES sea-going workshops on board research vessels were organized by WGPDMO in 1984 (southern North Sea), 1988 (Kattegat), 1994 (Baltic Sea, co-sponsored by the Baltic Marine Biologists, BMB), and 2005 (Baltic Sea) in order to intercalibrate and standardize methodologies for fish disease surveys (Dethlefsen *et al.*, 1986; ICES, 1989, 2006a; Lang and Mellergaard, 1999) and to prepare guidelines. While first guidelines were focused on externally visible diseases and parasites, WGPDMO developed guidelines for macroscopic and microscopic inspection of flatfish livers for the occurrence of neoplastic lesions at a later stage. Further intercalibration and standardization of methodologies used for studies on liver pathology of flatfish were a major issue of the 1996 ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants (ICES, 1997). This formed the basis from which the BEQUALM programme developed for the application of liver pathology in biological effects monitoring (Feist *et al.*, 2004) (Table 11.3).

Table 11.3 BEQUALM categories of histopathological liver lesions in fish that should be used for the CEMP general and PAH-specific biological effects monitoring

HISTOPATHOLOGY CATEGORIES	HISTOPATHOLOGICAL LESIONS
Non-specific lesions	Coagulative necrosis, apoptosis, lipoidosis (microvesicular and macrovesicular steatosis), haemosiderosis, variable glycogen content, increased numbers and size of macrophage aggregates, lymphocytic/monocytic infiltration, granuloma, fibrosis, regeneration
Early toxicopathic non-neoplastic lesions	Phospholipidosis, fibrillar inclusion, hepatocellular and nuclear polymorphism, hydropic degeneration, spongiosis hepatis
Foci of cellular alteration	Clear cell foci, vacuolated foci, eosinophilic foci, basophilic foci, mixed cell foci

Benign neoplasms	Hepatocellular adenoma, cholangioma, haemangioma, pancreatic acinar cell adenoma
Malignant neoplasms	Hepatocellular carcinoma, cholangiocarcinoma, pancreatic acinar cell carcinoma, mixed hepatobiliary carcinoma, haemangiosarcoma, haemangiopericytic sarcoma

A fish disease database has been established within the ICES Data Centre consisting of disease prevalence data of key fish species and accompanying information submitted by ICES Member Countries. Submission of fish disease data to the ICES Data Centre has been formalized by the introduction of the ICES Environmental Reporting Format designed specifically for this purpose. This is used for fish disease, contaminant, and biological effects data. The programme includes internal screening procedures for the validation of the data submitted, providing further quality assurance.

The ICES fish disease database is extended on an annual basis to include data from other species and areas within the OSPAR maritime area as well as data on studies into other types of diseases (e.g. macroscopic liver neoplasms and liver histopathology). To date, the data mainly comprise information from studies on the occurrence of externally visible diseases and macroscopic liver lesions in the common dab (*Limanda limanda*) and the European flounder (*Platichthys flesus*) from the North Sea and adjacent areas, including the Baltic Sea, Irish Sea, and the English Channel. In addition, reference data are available from pristine areas, such as waters around Iceland. In total, data on length, sex, and health status of more than 700 000 individual specimens, some from as early as 1981, have been submitted to ICES, as well as information on sampling characteristics (Wosniok *et al.*, 1999; Lang and Wosniok, 2008).

Current ICES WGPDMO activities have focused on the development and application of statistical techniques for an assessment of disease data with regard to the presence of spatial and temporal trends in the North Sea and western Baltic Sea (Wosniok *et al.*, 1999; Lang and Wosniok, 2008). An output of WGPDMO's activities is the ICES web-based report on wild fish diseases, consisting of trend maps and associated information. In a more holistic approach, pilot analyses have been carried out combining the disease data with oceanographic, nutrient, contaminant, and fishery data extracted from the ICES Data Centre in order to improve the knowledge about the complex cause–effect relationships between environmental factors and fish diseases (Lang and Wosniok, 2000; Wosniok *et al.*, 2000). These analyses constituted one of the first attempts to combine and analyse ICES data from various sources and can, therefore, be considered as a step towards a more comprehensive integrated assessment.

Quality assurance is in place for externally visible diseases, macroscopic liver neoplasms, and liver histopathology via the ongoing BEQUALM programme (additional information under "Assessment of thresholds" below). Regular intercalibration and ring-test exercises are conducted. The basis for QA procedures is provided in two key publications in the *ICES TIMES* series (Bucke *et al*., 1996; Feist *et al*., 2004) and a BEQUALM CD-ROM of protocols and diagnostic criteria and reporting requirements for submission of data to ICES.

11.4 Review of the environmental variables that influence fish diseases and liver pathology

The multifactorial aetiology of diseases, in this context in particular of externally visible diseases, is generally accepted. Therefore, externally visible disease has correctly been placed into the general biological effect component of the OSPAR CEMP. Most wild fish diseases monitored in past decades are caused by pathogens (viruses, bacteria). However, other endogenous or exogenous factors may be required before the disease develops. One of these factors may be environmental pollution, which may either affect the immune system of the fish in a way that increases its susceptibility to disease, or may alter the number and virulence of pathogens. In addition, contaminants may also cause specific and/or non-specific changes at various levels of biological organization (molecule, subcellular units, cells, tissues, organs) leading to disease without involving pathogens.

The occurrence of significant changes in the prevalence of externally visible fish diseases can be considered a non-specific and more general indicator of chronic rather than acute (environmental) stress, and it has been speculated that they might, therefore, be an integrative indicator of the complex changes typically occurring under field conditions rather than a specific marker of effects of single factors. Because of the multifactorial causes of externally visible diseases, the identification of single factors responsible for observed changes in disease prevalence is difficult, and scientific proof of a link between contaminants and externally visible fish diseases is hard to achieve. Nevertheless, there is a consensus that fish disease surveys should continue to be part of national and international environmental monitoring programmes since they can provide valuable information on changes in ecosystem health and may act as an "alarm bell" potentially initiating further more-specific studies on cause-and-effect relationships.

In the statistical analysis of ICES data on externally visible diseases (lymphocystis, epidermal hyperplasia/papilloma, acute/healing skin ulceration) of dab from different North Sea regions, it could be demonstrated that there were significant spatial differences, both in terms of absolute levels and temporal changes in disease prevalence in the North Sea. While data from the 1990s revealed stable or decreasing disease prevalence in the majority of sampling sites, some areas in the North Sea showed increasing trends for some of the diseases, indicating a change in environmental conditions adversely affecting the health status of dab (Wosniok *et al.*, 1999). The results from the subsequent multivariate analysis on the relationship between the prevalence of the diseases with potentially explanatory environmental and host-specific factors (also extracted from the ICES fishery, oceanography, and environmental databases) clearly highlighted the multifactorial aetiology of the diseases under study. A number of natural and anthropogenic factors (stock composition, water temperature, salinity, nutrients, contaminants in water, sediments, and biota) were found to be significantly related to the temporal changes in disease prevalence. However, depending on area, time range, and data availability, different sets of factors were identified. This reflects the multifactorial aetiology of the diseases covered, but was also attributed to some high correlations among the explaining quantities (Lang and Wosniok, 2000; Wosniok *et al.*, 2000).

The presence of macroscopic liver neoplasms and certain types of histopathological liver lesions is a more direct indicator of contaminant effect and has been used for many years in environmental monitoring programmes around the world. Liver neoplasms (either detected macroscopically or by histopathological analysis) are likely to be associated with exposure to carcinogenic contaminants, including PAHs, and are, therefore, considered appropriate indicators for general and for PAH-specific biological effects monitoring. Therefore, monitoring of macroscopic liver neoplasms in the CEMP should not only be part of the CEMP general biological effects monitoring, but also of the CEMP PAH-specific biological effects monitoring.

The study of liver histopathology comprises the detection of more lesion categories (non-specific, neoplastic, and non-neoplastic toxicopathic lesions), reflecting responses to a wider range of contaminants (including PAHs), but also to other environmental stressors and is, therefore, considered an appropriate indicator for both general and PAH-specific biological effects monitoring.

The liver is the main organ involved in the detoxification of xenobiotics, and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and representative of biological endpoints of contaminant exposure (Myers *et al.*, 1987, 1992, 1998; Stein *et al.*, 1990; Vethaak and Wester, 1996; Stentiford *et al.*, 2003; Feist *et al.*, 2004). The majority of lesions observed in field-collected animals have also been induced experimentally in a variety of fish species exposed to carcinogenic compounds, PAHs in particular provide strong supporting evidence that wild fish exhibiting these lesions could have been exposed to such environmental contaminants.

11.5 Assessment of the thresholds when the response (prevalence and incidence of fish disease) can be considered to be of concern and/or require a response

As indicated above, ICES has developed requirements for the international reporting of fish diseases over many years in order to minimize variation between laboratories regarding the accuracy and reproducibility of data generated. These have been reviewed by BEQUALM and produced in CD-ROM format. Each grossly visible disease (lymphocystis, acute and healing skin ulcerations, epidermal hyperplasia/ papilloma, and liver nodules, etc.) has a minimum number of examined individuals requirement for reporting. Severity is assessed according to criteria allocated to three stages (lymphocystis, ulcerations, and epidermal hyperplasia/papilloma only). Macroscopic liver neoplasms are only recorded if the minimum diameter exceeds 2 mm. Each case has to be verified histologically to exclude the possibility that the macroscopic lesion is the response to parasites, cysts, necrotic or inflammatory foci. As such, the acceptable limits of variation for disease recording are well established.

With regard to the application of liver histopathology as a tool in biological effects monitoring, the activities undertaken in ICES and within BEQUALM have been successful in the establishment of the methodology and diagnostic criteria. The diagnostic key provides clear criteria to discriminate between the lesion types, thus minimizing the possibility of misdiagnosis. Ring-tests and other intercalibration exercises are regularly undertaken in order to minimize interobserver variation and to establish acceptable limits of variation. These are carried out as an ongoing process in order to ensure continuous quality assurance of data obtained.

These quality assurance procedures implemented are a crucial prerequisite for the establishment of assessment criteria and reference or threshold values applied by all institutions involved in fish disease monitoring in order to take decisions on further actions. The ICES WGPDMO and the 2009 ICES/OSPAR WKIMC addressed the question of establishing background/reference levels of disease and criteria for their assessment (see Section 11.6).

11.6 Proposals for assessment tools

The development of assessment tools for externally visible diseases, macroscopic neoplasms, and liver histopathology has been carried out by the ICES WGPDMO (ICES, 2006b, 2007, 2008, 2009, 2011). Further additions were proposed at the 2009 ICES/OSPAR WKIMC.

The ICES WGPDMO developed a fish disease index (FDI) using data on diseases of the common dab (*Limanda limanda*) as a model, the aim of which is to summarize information on the disease status of individual fish into one robust and easy-to-understand and easy-to-communicate numeric figure. By applying defined assessment criteria and appropriate statistics, the FDI can be used to assess the level and temporal changes in the health status of fish populations and can, thus, serve as a tool for the assessment of the ecosystem health of the marine environment (e.g. related to the effects of anthropogenic and natural stressors). Its design principle allows the FDI to be applied to other species with other sets of diseases. Therefore, the FDI approach is applicable for wider geographical areas (e.g. as part of the convention-wide OSPAR monitoring and assessment programme).

For the calculation of the FDI, the following components are required:

- data on diseases of the common dab (*Limanda limanda*) (can be adapted to other fish species, provided that sufficient appropriate data are available);
- information on the presence or absence of a range of diseases monitored on a regular basis, categorized as externally visible diseases (EVD: nine key diseases, including three parasites), macroscopic liver neoplasms (MLN: two key diseases) and non-specific and contaminant-specific liver histopathology (LH: five key diseases) (see Table 11.4);
- for most diseases, data on three severity grades (reflecting a light, medium, or severe disease status);
- disease-specific weighting factors, reflecting the impact of the diseases on the host (assigned based on expert judgements);
- adjustment factors for effects of size and sex of the fish as well as for season effects. For macroscopic liver neoplasms and liver histopathology, age will be considered as well.

Table 11.4. Disease categories and key diseases to be used for calculating the fish disease index for dab (*Limanda limanda*). ICES (2009)

Externally visible diseases	Liver histopathology: (a) non-specific lesions	Liver histopathology: (b) contaminant-specific lesions	Macroscopic liver neoplasms
Lymphocystis	Non-specific lesions	Early non-neoplastic toxicopathic lesions	Benign neoplasms
Epidermal hyperplasia/papilloma	(see Table 11.3)	Pre-neoplastic foci of cellular alteration (FCA)	Malignant neoplasms
Acute/healing skin ulceration		Benign neoplasms	(see Table 11.3)
X-cell gill disease		Malignant neoplasms	
Hyperpigmentation		(see Table 11.3)	
Acute/healing fin rot/erosion			
Stephanostomum baccatum			
Acanthochondria cornuta			
Lepeophtheirus pectoralis			

The result of the calculation is a FDI value for individual fish which is scaled in such a way that values can range from 0 to 100, with low values representing healthy and high values representing diseased fish. The maximum value of 100 can only be reached in the (purely theoretical and unrealistic) case that a fish is affected by all diseases at their highest severity grades. From the individual FDIs, mean FDIs for a sample from a fish population in a given sampling area can be calculated. Usually a sample in the present sense consists of the data collected in an ICES statistical rectangle during one cruise, but other geographical entities may be used as well. All assessments are based on mean FDI values calculated from these samples. Depending on the data available, FDIs can be calculated either for single disease categories or for combinations thereof.

The assessment of the mean FDI data considers (a) long-term FDI level changes, (b) FDI trends in the recent five-year time-window, and (c) comparing each FDI to its BAC and EAC where these are defined. While assessments (a) and (b) are done on a region-wise basis, global BAC and EAC are used for assessment (c). The assessment approaches (a) and (b) do not apply any global background or reference values or assessment criteria as is often done for chemical contaminants or for biochemical biomarkers. Instead, these assessment approaches use the development of the mean FDI within the geographical units (usually ICES rectangles) over a given period of time, based on which region-specific assessment criteria are defined. The reason for choosing this approach is the known natural regional variability of the disease prevalence (even in areas considered to be pristine), making it difficult to define generally applicable background/reference values that can uniformly be used for all geographical units to be assessed. This approach is based on the availability of disease data over a longer period of time (ideally 10 observations, e.g. in the case of biannual monitoring over a period of five years) for every geographical area to be assessed. The assessment approach (c) ignores the known regional differences and involves globally defined assessment criteria, with the consequence that within-region variation might be dominated by general differences in regional levels. However, using assessment approach (c), the FDI can also be used for exploratory monitoring in areas not studied before or for newly installed fish disease monitoring programmes after some modification.

Formally, the FDI is a weighted sum of disease occurrence observed for individual fish. Diseases may be recorded graded or ungraded (regarding the severity/intensity of the disease), and various sets of diseases can be used. Weights result from expert judgement and disease grade. The effect of sampling fluctuations in length (in case of macroscopic liver neoplasms and liver histopathology also age), sex, and effects of the sampling season are removed by applying adjustment factors. From the adjusted FDI, the BAC are defined as the 10th percentile of the FDI sample means, weighted by the number of cases in the sample. EAC are derived from the relationship between FDI sample means and condition factor (CF) means of the fish. This relation is described by linear regression from which the FDI associated with a 10% loss in CF compared with the mean CF for FDI = 0 is calculated. This value is the EAC, which exists only if the slope of the regression line is negative and the regression line intersects the line of 10% loss in the admissible FDI range (i.e. between 0 and 100). The procedure for calculating BAC and EAC is applied separately to the various FDI versions. These differ by either using ungraded or graded diseases and by using different collections of diseases.

The final products of the assessment procedure are:

- graphs showing the temporal changes in mean FDI values in a geographical unit over the entire observation period;
- maps in which the geographical units assessed are marked with green, yellow, or red smiley faces, indicating long-term changes (e.g. comparing the past five years to the preceding five-year period) in health status of the

fish population (green, improvement of the health status; yellow, indifferent variation; red, worsening of the health status, reason for concern, and motivation for further research on causes);

- maps in which the geographical units assessed are marked with green, yellow, or red smiley faces, indicating trends in health status of the fish population during the past five years (green, improvement of the health status; yellow, indifferent variation; red, worsening of the health status, reason for concern, and motivation for further research on causes); and
- maps in which the geographical units assessed are marked with green, yellow, or red smiley faces, indicating the level of the FDI observed at a defined point in time (green, FDI level ≤ BAC; yellow, BAC < FDI level ≤ EAC; red, EAC < FDI level). The red colour signals either population effects of disease (externally visible fish diseases, non-specific liver histopathology) or unacceptable contaminant effects (contaminant-specific liver histopathology, macroscopic liver neoplasms).

The ICES WGPDMO applied the FDI approach and the assessment for the common dab from the North Sea using ICES fish disease data extracted from the ICES Environmental Data Centre twice in 2008 and, using an extended dataset, in 2009 (ICES, 2008, 2009). The results have been included in the OSPAR QSR 2010 (OSPAR, 2010).

At the 2009 ICES/OSPAR WKIMC, concepts for developing assessment criteria (BAC/EAC) for macroscopic liver neoplasms and for non-specific and contaminant-specific liver histopathology were proposed and were later further elaborated by WGPDMO (ICES, 2011, 2012). These are provided in Table 11.1, which also contains information on the BAC and EAC for the externally visible disease. BAC and EAC values for different combinations of externally visible diseases of dab reviewed by ICES WGPDMO (ICES 2012) are provided in Table 11.2. BAC and EAC values for non-specific and contaminant-specific liver histopathology, as well as for macroscopic liver neoplasms, have not yet been calculated and are awaiting actual ICES data.

11.7 Final remarks

Some amendments still need to be made by OSPAR in the JAMP Guidelines for general and for PAH-specific biological effects monitoring and the terminology used therein:

- In the JAMP Guidelines for PAH-specific biological effects monitoring, chapter 4.1 and 5, the term ″Liver pathology″ should be changed to ″Liver histopathology″ and the term ″external diseases″ should be changed to ″externally visible diseases″ since these terms more correctly describe the technique to be applied.
- In the table of contents of the JAMP Guidelines for PAH-specific biological effects monitoring, the terms ″histopathology″ and ″liver pathology″ should be replaced by ″liver histopathology″ since this term more correctly describes the technique to be applied.

12 Background document: intersex (ovotestis) measurement in marine and estuarine fish

Grant D. Stentiford

12.1 Summary

Applicability across the OSPAR maritime area. The presence of susceptible host species utilized in monitoring programmes in marine and estuarine habitats of the OSPAR region make this an applicable measurement in field programmes. The requirement for the sampling of testis from male fish captured in such programmes and the assessment of these tissues by histology can be aligned with the sampling of other tissues currently assessed for fish disease work (e.g. for liver cancer assessment). The epidemiological basis for the sampling of fish for intersex measurement is, therefore, aligned with other field sampling programmes for fish health.

Status of quality assurance. Formal quality assurance (QA) for the measurement of intersex in marine and estuarine fish has not been carried out under existing programmes (such as BEQUALM), but published methods are available for the grading of intersex severity in flatfish collected from monitoring programmes. These methods would be directly applicable to QA programmes. The sampling of materials from epidemiologically relevant numbers of animals is also well characterized in the literature and is outlined in this document.

Influence of environmental variables. Although sex determination can be influenced by environmental factors and age, there has been an historic linkage between sites with the highest prevalence of intersex fish, biomarkers for exposure to endocrine-disrupting chemicals (e.g. vitellogenin), and anthropogenic contaminants known to elicit development of ovotestis in a range of test species.

Assessment of thresholds. Threshold assessment to indicate an affected site has not previously been discussed for measurement of intersex (ovotestis) in male fish. However, based upon the reported prevalence of the condition in marine and estuarine fish from the OSPAR region, and the constraints inherent with the sampling of large populations for health effects, it would appear that a threshold of 5% prevalence (in external males) may be used to indicate impact. The epidemiological basis for this is discussed in this document.

Proposals for assessment tools. Given background data on QA techniques for intersex measurement, it seems appropriate to propose a two-tier assessment tool. Tier 1 consists of an individual sample grading system for intersex severity based on the methodology presented by Bateman *et al.* (2004). Tier 2 consists of apparent prevalence estimates based upon a sampling regime designed to detect a 5% prevalence of intersex at 95% confidence. Both of these tools can be combined to provide a population-level and individual-level assessment tool for the condition. Because intersex prevalence is likely to be negligible in non-affected populations, survey designs are likely to be similar to that for fish disease measurement, whereby detection is based upon diseases present in a population at 5% prevalence (95% confidence). In this way, >5% prevalence would be considered the cut-off point for definition of an affected population. The use of cohort-matching, similar to that for assessment of liver pathology in flatfish, is recommended to remove any confounding effects of age on intersex prevalence (e.g. use of fish 4 years old; Stentiford *et al.*, 2010).

12.2 Assessment of the applicability of intersex measurement across the OSPAR maritime area

In recent years, a significant proportion of research into the biological effects of contaminants in the aquatic environment has been devoted to the study of endocrine-disrupting chemicals (EDCs) of anthropogenic origin. EDCs have been widely reported to impair fertility, development, growth, and metabolism in a range of animal groups (see Colborn *et al*., 1996). The effects of exposure of fish to such compounds include disturbed maturation and degeneration of the gonads, elevated concentrations of vitellogenin (egg-yolk protein) in the plasma of male fish, and the presence of intermediate or ″intersex″ gonads (Gimeno *et al*., 1996). Using histological analysis, fish with the intersex condition are seen to possess oocytes within their normal testicular matrix (Sharpe, 1997; Bateman *et al*., 2004). Until the early 1990s, intersex had only rarely been described from fish in the wild (Jafri and Ensor, 1979; Slooff and Kloowijk-Vandijk, 1982; Blachuta *et al*., 1991). However, the condition has now been detected in several wild freshwater and migratory species, including roach (*Rutilus rutilus*; Jafri and Ensor, 1979; Purdom *et al*., 1994; Jobling *et al*., 1998), gudgeon (*Gobio gobio*; van Aerle *et al*., 2001), barbel (*Barbus plebejus*; Vigano *et al*., 2001), chub (*Leuciscus cephalus*; Minier *et al*., 2000), bream (*Abramis brama*; Slooff and Kloowijk-Vandijk, 1982), white perch (*Morone americana*; Kavanagh *et al*., 2002), stickleback (*Gasterosteus aculeatus*; Gercken and Sordyl, 2002), coregonids (Mikaelian *et al*., 2002), grayling (*Thymallus thymallus*; Blachuta *et al*., 1991), and Atlantic salmon (*Salmo salar*; authors' pers. obs.). Furthermore, detection of elevated prevalences of intersex in some estuarine and marine species such as the European flounder (*Platichthys flesus*; Allen *et al*., 1999a), Japanese flounder (*Pleuronectes yokohamae*; Hashimoto *et al*., 2000), bothid flounder (*Bothus pantherinus*; Amaoka *et al*., 1974), common eel (*Anguilla anguilla*; Peters *et al*., 2001), and viviparous blenny (*Zoarces viviparus*; Matthiessen *et al*., 2000; Stentiford *et al*., 2003) suggest that the effects of anthropogenic EDCs may extend beyond inland river systems to coastal and even offshore waters. This is supported by reports of elevated plasma vitellogenin and ovotestis in male Mediterranean swordfish (*Xiphias gladius*; Fossi *et al*., 2001; Metrio *et al*., 2003) and the dab (*Limanda limanda*; Scott *et al*., 2007; Stentiford and Feist, 2005). In terms of species of relevance to the OSPAR region, those in which intersexuality (ovotestis) have been described from marine and estuarine habitats include flounder (Allen *et al*., 1999b; Stentiford *et al*., 2003; Bateman *et al*., 2004), dab (Stentiford and Feist, 2005), viviparous blenny (Stentiford *et al*., 2003; Lyons *et al*., 2004b), red mullet (Martin-Skilton *et al*., 2006), and the three-spined stickleback (Gercken and Sordyl, 2002).

12.3 Status of QA techniques for intersex measurement in marine and estuarine fish

Male fish with the intersex condition are seen to possess, at varying degrees of severity, oocytes within the testis; this being regarded as a phenotypic endpoint of endocrine disruption (both natural and anthropogenic) in male fish. Owing to the fact that the testis may appear normal from external observations, histological examination of the testis is necessary to identify and grade individual cases of intersex and to estimate prevalence in a population. Intersex has been recorded histologically in all of those species listed above as relevant to marine and estuarine waters from the OSPAR region. It is important to consider quality assurance techniques for intersex measurement at two levels: (i) individual (grading of intersex severity) and (ii) population (intersex prevalence).

12.4 Individual-level grading of intersex (ovotestis)

The most comprehensive assessment of ovotestis severity at the individual level has been presented by Bateman *et al*. (2004) for the European flounder. In this case, the study provided information on the different pathological manifestations of the intersex condition in flounder sampled from various estuarine and coastal waters of the UK and, furthermore, described the development and application of an ovotestis severity index (OSI), calculated for individual histological sections of gonad. The development of this index provides pathologists with a robust tool for the grading of the intersex condition in flounder and potentially other fish species sampled in the OSPAR region.

The study by Bateman *et al*. (2004) utilized samples collected from monitoring programmes around the UK over a four-year period (1998–2002) and assessed externally classified male flounder of above 15 cm in length. For histology, whole gonads were removed and fixed in a 10% solution of neutral buffered formalin prior to processing to wax using standard protocols. In order to assess the distribution of oocytes throughout the testis, all specimens examined were step-sectioned longitudinally at 0.2-mm intervals throughout tissue at a thickness of 3–5 µm, mounted onto glass slides, and stained using haematoxylin and eosin (H and E). Sections were analysed by light microscopy. In all 56 intersex cases were examined. All gonadal sections were viewed at low magnification using a 10× eyepiece and 10× objective lens, giving a total magnification of 100. Each gonadal tissue section was divided into a variable number of fields of view, depending on the size of the sample. The number of fields of view comprising the whole tissue section was then used to construct a virtual grid, with each square on the grid corresponding to a field of view. Only fields of view that contained 100% tissue coverage were included in calculations of the OSI. Each field of the grid was then scored for the presence of oocytes, the distribution of these oocytes, and their stage of development (according to previously published criteria in other fish species). The overall OSI takes into consideration both the oocyte development stages present and their distribution throughout the testis (see Figure 12.1 from Bateman *et al*., 2004).

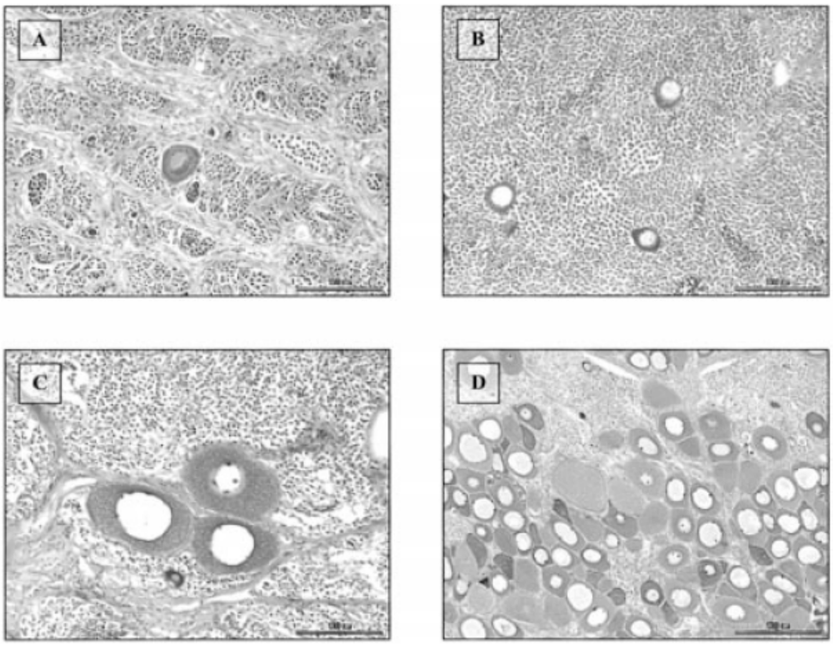


Figure 12.1. Oocyte distribution patterns in ovotestis cases. (A) Focal, only single oocytes are present within field of view. (B) Diffuse distribution, more than one oocyte is present in a field of view and are not closely associated with neighbouring oocytes. (C) Cluster distribution with more than one, but less than five, closely associated oocytes present within a field of view. (D) Zonal distribution, indicated by the presence of more than five closely associated oocytes within a field of view. In this case, oocytes in various stages of maturity can be seen. Haematoxylin and eosin stained sections. Scale bars = 100 μm.

In order to calculate the severity of the intersex condition within an individual section of gonadal material, an algorithm was formulated, incorporating the scores for development and distribution of oocytes within individual fields of view. This algorithm allowed for the calculation of the OSI for an individual section of gonad. The OSI was calculated as follows, where D_1 is the most advanced development stage of oocytes within a field of view (score 1–5), D_2 is the distribution of oocytes within a field of view (score 1–4), and X is the total number of fields of view examined.

$$\text{OSI} = \left(\frac{\sum [D_1 \cdot D_2]}{X} \right)$$

The OSI is a sum of the severity staging for each field of view in a section of gonad. By dividing this sum by the total number of fields of view in the whole section, the mean ovotestis severity per field of view can be obtained. For intersex flounder, this gives an overall OSI of >0 up to 20 (the maximum score, whereby each field of view contains over five vitellogenic oocytes in a zonal distribution). Testing this scoring system on field-collected samples of flounder, Bateman *et al*. (2004) used the OSI scores from each gonad to create a broad grading system of: absent (OSI = 0), stage 1 (OSI >0–5), stage 2 (OSI >5–10), and stage 3 (OSI >10–20). This was summarized in Table 12.1.

Table 12.1. Ovotestis ranking by stage based on histological appearance, proportion of fields containing oocytes, and the distribution and developmental stage of oocytes

Severity category	Histology	Proportion of fields of view with oocytes	Distribution and developmental stage of oocyte
Absent (score 0)	Testis structure is normal, with no oocytes present in section		
Stage 1 (score >0–5)	Structure of the majority of the testis appears normal	Generally below 50%	Single or multiple previtellogenic oocytes. Cortical alveolar or fully vitellogenic oocytes rarely are present
Stage 2 (score >5–10)	Regions of the testis are altered, replacement of testicular material with oocytes	Up to 75%	Majority of oocytes are previtellogenic, present in clusters or zones in high proportion of fields of view. Single or multiple vitellogenic oocytes
Stage 3 (score >10–20)	Majority of testis is disrupted, replacement of testicular material with oocytes in various stages of development	Above 75%	Associated previtellogenic or vitellogenic oocytes through majority of section

12.5 Population prevalence of intersex (ovotestis)

The second level of assessment of intersex (ovostestis) in marine and estuarine fish from the OSPAR region requires an indication of prevalence (or the total number of cases in the population, divided by the number of individuals in the population). Because it is problematic to define the number of individuals in a wild population of marine or estuarine fish, the estimation of prevalence (or so-called apparent prevalence) is, therefore, carried out by sampling a statistically significant number of animals from a population exceeding a presumed size (e.g. >10 000 individuals). The size of the sample required will also depend on the necessity of detecting a given prevalence (e.g. 1, 2, 5%) and the confidence level of detecting this prevalence (e.g. 90, 95, 99%). Although the majority of studies examining the presence of intersex in wild populations do not appear to have followed statistical guidelines relating to the sampling of wild populations (e.g. see Simon and Schill, 1984), it is perhaps relevant that the approach to monitoring for intersex should follow that outlined in the chapter for fish diseases and as reported in studies such as those of Stentiford *et al.* (2009, 2010). In this context, sampling is designed to detect a disease prevalence of 5% at a confidence level of 95%. Using these figures, 59 individuals should be sampled if the population size is assumed to be 10 000 individuals. By using the same confidence of detecting lower prevalence of intersex, sample sizes would need to increase to 148 individuals (for 2% prevalence) and 294 individuals (for 1% prevalence). It should be noted, however, that where populations exceed 100 000, 500 000 or 1 000 000 individuals, sample sizes required to detect a 5, 2, and 1% prevalence at 95% confidence are considerably larger (597, 1494, and 2985 individuals, respectively). Clearly, cost and conservation limitations will relate to most monitoring schemes so that these latter numbers become somewhat unfeasible. It is for this reason that presuming a population size of 10 000 and sampling to detect 5% prevalence at 95% confidence has been chosen for much of the fish disease work (Feist *et al.*, 2004).

When considering apparent prevalence of intersex in a population of marine or estuarine fish sampled from the OSPAR region, it is useful to consider the reported prevalence range for the condition in relevant species. Available data for the key monitoring species are as follows:

Flounder (*Platichthys flesus*)	Up to 20% (Allen *et al.*, 1999a)
	Up to 9% (Allen *et al.*, 1999b)
	Up to 8% (Minier *et al.*, 2000)
	Up to 8.3% (Stentiford *et al.*, 2003)
Viviparous blenny (*Zoarces viviparus*)	Up to 27.8% (Gercken and Sordyl, 2002)
	Up to 25% (Stentiford *et al.*, 2003)
Dab (*Limanda limanda*)	Up to 14.3% (Stentiford and Feist, 2005)
Red mullet (*Mullus barbatus*)	Up to 14.3% (Martin-Skilton *et al.*, 2006)
Stickleback (*Gasterosteus aculeatus*)	Up to 12.5% (Gercken and Sordyl, 2002)

Given that intersex appears to exist at a range of between 0 and 27.8% in different monitoring species, a sampling regime based upon detection of 5% prevalence at 95% confidence appears appropriate. Furthermore, multisite work in several species [e.g. flounder and dab by Stentiford *et al.* (2003, 2005), respectively] has demonstrated that intersex is detected at some sites and not at others when this regimen is utilized. This indicates that intersex, if present, occurs at below 5% at these latter sites. As such, for monitoring purposes, it could be proposed that 5% prevalence of intersex is considered to be "above baseline", with all sites with a prevalence above this being further assessed for intersex severity using the OSI approach of Bateman *et al.* (2004). This gives a two-tiered assessment of intersex utilizing apparent prevalence in the population, and an indicator for severity in affected individuals.

12.6 Review of the environmental variables that influence the presence of intersex in marine and estuarine fish

Although the link between the formation of intersex (ovotestis) and exposure to anthropogenic contaminants considered to be "endocrine disruptors" has been demonstrated for several fish species (e.g. Gimeno *et al.*, 1996, 1997), it is also known that intersex and sex reversal are not specific markers for oestrogens, but rather they have many causes (including androgens, aromatase inhibitors, and even water temperature shifts). Recent work has also demonstrated a potential for age to affect the occurrence and prevalence of the condition in freshwater fish species (Jobling *et al.*, 2009). For certain species utilized in monitoring programmes in the OSPAR region, there is a clear historical link between those sites where anthropogenic endocrine disruptors, direct biomarkers of endocrine disruption (e.g. vitellogenin, Vtg), and the presence of intersex in populations residing in those habitats are most pronounced (for example, see links between papers by Allen *et al.*, 1999a,b and Stentiford *et al.*, 2003 for estuarine flounder). Extending this relationship between cause and effect to offshore populations is not so clear, although data presented by Scott *et al.* (2007) showing elevated Vtg in dab sampled from certain North Sea sites do correspond to data presented by Stentiford and Feist (2005) for intersex in the same species from these sites. Complications in specifically linking the presence of a chronic marker (such as intersex) with more acute phase markers (such as Vtg), or the burden of anthropogenic chemicals, are not unique in this instance, with similar parallels being reported in liver cancers present in a consistent, but as yet unexplainable manner in multiyear samples of dab collected from offshore sites (Stentiford *et al.*, 2009, 2010). Interestingly, those estuarine and offshore sites with the highest prevalence of liver pathologies (including cancer) are also those where intersex have been reported. However, because hatchlings and juveniles are likely to inhabit different grounds to those where adults are sampled (Dipper, 1987), and it is at these early life stages at which sex is determined (and at which disruption may occur; Gimeno *et al*., 1997; Devlin and Nagahama, 2002), the presence of fish with the intersex condition at the particular offshore sites may not necessarily reflect the presence of EDCs at the site, but rather their presence at sites where hatching and early growth occurs. Future studies should be directed towards the measurement of intersex in fish of known age, or in earlier life stages residing at monitoring sites and at those sites identified at nursery grounds for the key monitoring species. Comparisons of the prevalence of the intersex condition in juvenile and adult fish of the same species may furthermore provide clarification on the population-level effects of EDCs in the marine environment and on their long-term ecological effects on sensitive ecosystems. Coupled with studies on the population genetics of these species and the identification of specific spawning grounds for different adult stocks, the potential selective pressure imposed by endocrine disturbances may also be identified.

12.7 Assessment of the thresholds when the response (prevalence of intersex) can be considered to be of concern and/or require a response

As stated above, given that intersex appears to exist at a range of 0–27.8% in different monitoring species, a sampling regime based upon detection of 5% prevalence at 95% confidence appears appropriate. Furthermore, multisite surveys in several species [e.g. flounder and dab by Stentiford *et al*. (2003) and Stentiford and Feist (2005), respectively] have demonstrated that intersex is detected at some sites and not at others when this regimen is utilized. This indicates that intersex, if present, occurs at below 5% at these latter sites. As such, for monitoring purposes, it could be proposed that 5% prevalence of intersex is considered to be ”above baseline”, with all sites with a prevalence above this being further assessed for intersex severity using the OSI approach of Bateman *et al*. (2004). This gives a two-tiered assessment of intersex utilizing apparent prevalence in the population, and an indicator for severity in affected individuals. It also allows for the discounting of potential isolated cases of intersex that may occur as a result of genetic abnormalities or other causes.

12.8 Proposals for assessment tools

Given background data on quality assurance techniques for intersex measurement, it seems appropriate to propose a two-tier assessment tool. Tier 1 consists of an individual sample grading system for intersex severity based on the methodology presented by Bateman *et al*. (2004). Tier 2 consists of apparent prevalence estimates based upon a sampling regime designed to detect a 5% prevalence of intersex at 95% confidence. Both of these tools can be combined to provide a population-level and individual-level assessment tool for the condition. Because intersex prevalence is likely to be negligible in non-affected populations, survey designs are likely to be similar to that for fish disease measurement, whereby detection is based upon diseases present in a population at 5% prevalence (95% confidence). In this way, >5% prevalence would be considered the cut-off point for definition of an affected population. It is recommended that cohort-matching is applied when comparing fish captured from different geographic sites, similar to the manner carried out for assessment of liver pathologies (Stentiford *et al*., 2010).

13 Background document: reproductive success in eelpout (*Zoarces viviparus*)

Jakob Strand

13.1 Background

The eelpout (*Zoarces viviparus*; Figure 13.1), also called viviparous blenny, can be used as a bioindicator of the impact of hazardous substances on reproductive success of viviparous fish in the marine environment. The reproductive success in fish is a generic "stress" indicator; causal agents may, however, be identified through a combination of chemical analyses of fish tissue, a knowledge of the history of contamination of the local environment to which the fish have been exposed, and/or follow-up laboratory experimentation (Jacobsson *et al*., 1986). Substances such as organochlorines, pesticides, polycyclic aromatic hydrocarbons (PAHs), heavy metals, and organometals can affect embryo and larval development in fish (Bodammer, 1993). Several of these substances, which may induce developmental, morphological, and/or skeletal anomalies, have also been identified as endocrine-disrupting substances (Davis, 1997).

Figure 13.1. The eelpout is a viviparous fish and the pregnant female bears 20–300 living embryo and larvae in the ovarian cavity (Photo: Jakob Strand).

The eelpout inhabits coastal waters from the White Sea to the southern North Sea. However, it is not equally abundant in all areas and it may, therefore, be difficult to sample adequate numbers throughout the OSPAR area. Use in regional assessments is more appropriate. However, studies of reproductive success in eelpout are recommend by ICES, OSPAR, and HELCOM for marine monitoring programmes of biological effects (OSPAR, 1997; ICES, 2004; HELCOM, 2006), and, for instance, Sweden and Denmark have included this method in regional and national monitoring programmes in coastal waters of the Baltic Sea, the Kattegat, and the Skagerrak.

It should be noted that eelpouts are protected in the pregnancy period in some areas, and an official sampling licence for monitoring activities should be obtained, where necessary.

The methodology is well defined for studies in coastal waters, and national guidelines exist (Jacobsson *et al*., 1986; Neuman *et al*., 1999; Strand and Dahllöf, 2005).

An international guideline is in preparation and will be published in the *ICES TIMES* series. As method quality assurance, some international and national workshops have been held in relation to the monitoring programmes (e.g. BEQUALM, 2000). A Baltic workshop has been held in 2009 as part of BONUS+ projects BALCOFISH and BEAST. National workshops in relation to NOVANA monitoring activities have also been held in Denmark (Strand, 2005a).

Elevated levels of adverse developmental effects of embryo and larvae in eelpout broods have been found in populations living in areas contaminated with effluents from cities and industry. In comparison, only low levels of such effects generally occur in populations living in areas regarded as reference sites (e.g. Vetemaa *et al.*, 1997; Ådjers *et al.*, 2001; Sjölin *et al.*, 2003; Strand *et al.*, 2004; Kalmarweb, 2005; Gercken *et al.*, 2006); however, some year-to-year variations can occur (Figure 13.2). Acute larval mortality has also been observed in eelpout exposed to pulp mill effluents (Jacobsson *et al.*, 1986). Other environmental stress factors, such as increased temperatures and oxygen depletion events, may, however, also affect eelpout reproduction (Vetemaa, 1999; Fagerholm, 2002; Strand *et al.*, 2004). Reproductive success in eelpout is regarded as a general (i.e. non-specific) biological indicator of impaired fish reproduction.

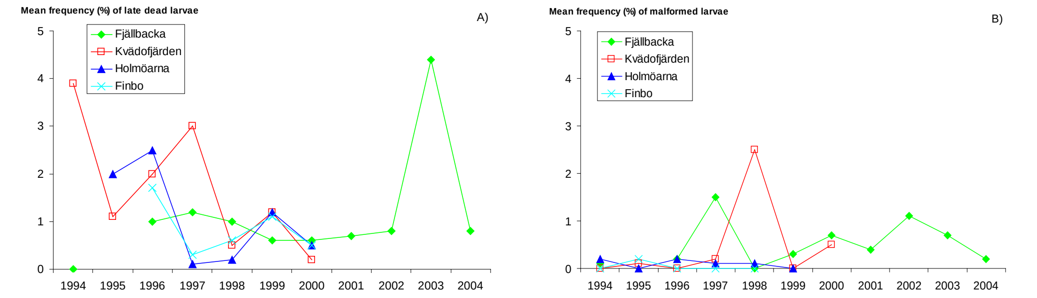


Figure 13.2. Year-to-year variations in mean frequencies of (A) late dead larvae and (B) malformed larvae at four Swedish monitoring stations regarded as reference sites.

According to the technical guidelines used in the Swedish and Danish monitoring programmes (Neuman *et al.*, 1999; Strand and Dahllöf, 2005), supporting parameters such as water temperature and salinity, together with general fish physiological and reproductive parameters, should be recorded when reproductive success in eelpout is examined.

For simplifying reasons and as a first step, only the occurrence of abnormal development of embryo and larvae in the broods of pregnant eelpouts has been included in the proposed assessment criteria for impaired reproduction. However, other relevant fish physiological and reproductive parameters must been seen as supplementary parameters, and their integration should be further evaluated.

Abnormal development of embryo and larvae in eelpout broods can, according to the Swedish and Danish guidelines (Neuman *et al.*, 1999; Strand and Dahllöf, 2005), be characterized as follows:

- Malformed larvae: larvae with morphological and/or skeletal gross anomalies. This includes yolk-sac or intestinal defects, bent spine or spiral shapes of the spinal axis, eye defects including rudimentary or missing eye(s), cranio-facial defects, and conjoined/Siamese twins more or less separated.

- Late dead larvae: dead larvae without malformations and with a length >15 mm (>10 mm in Denmark).
- Growth-retarded larvae: normal developed larvae which are smaller than the three highest length classes in the broods.

Less visible aberrations, including altered behavioural aspects, are not included in this analysis, although they can be highly ecological relevant effects.

Studies on skewed sex ratio in eelpout broods can be used as an indicator of endocrine disruptions. For instance, a Swedish study has found significant male-biased sex ratios of eelpout embryos (53.9–61.3% males) in an area contaminated with paper mill effluents (Larsson and Förlin, 2002). In eelpout broods, the reference conditions are supposed to be 50 : 50 between females and males.

13.2 Proposal for assessment criteria of the reproductive success in eelpout

The approach for deriving the assessment criteria is based on statistical analyses, which imply that the effect level must be significantly different from the background response (i.e. where the impact of environmental factors such as contaminants can be regarded as close to zero).

In all 52 datasets from 14 sampling stations regarded as reference sites and 41 datasets from 22 stations not regarded as reference sites in the Baltic Sea, the Kattegat, and the Skagerrak from the period 1994–2004 are available for the analyses. However, an important assumption is that adequate reference sites actually can be found in the Baltic Sea, the Kattegat, and the Skagerrak, although these waters are generally regarded to be more polluted than the North Sea and the North Atlantic.

Data related to frequencies (mean percents) of both abnormal larvae per female and of broods with >5% abnormal larvae (i.e. related to individual pregnant females) are used in the analyses. However, data of >5% distributions are only available from 37 of the 93 datasets, and there is no information available of broods with >5% growth-retarded larvae.

Data on frequencies of females with (at least one) abnormal larvae present in the brood are not included in this analysis, because the influence of brood size cannot be discriminated.

Proportion of abnormal larvae per female	Proportion of broods with elevated levels of abnormal larvae
Mean frequency of late dead larvae	Frequency of broods containing >5% late dead larvae
Mean frequency of malformed larvae	Frequency of broods containing >5% malformed larvae
Mean frequency of growth-retarded larvae	No data

In the assessment criteria, the upper level of the background response (class I) is determined by the 90th percentile of all datasets observed in areas regarded as reference sites (i.e. distant to larger cities and industry).

13.2.1 Assessment criteria related to mean frequencies of abnormal larvae in broods

Most studies on the development of eelpout embryo and larvae from the Baltic Sea, the Kattegat, and the Skagerrak have used mean frequencies of late dead, malformed, and growth-retarded larvae in the broods as a measure of impaired reproduction in eelpout.

In areas considered as reference sites, only small frequencies of abnormal larvae have been found, if any. Values of 90th percentiles have been found to be 1% malformed, 2% late dead, and 4% growth-retarded larvae, respectively.

Table 13.1. Proposal for assessment criteria for the mean frequencies of malformed larvae, late dead larvae and growth-retarded larvae per station

Assessment class	Class I Background response	Class II
Mean frequency of malformed larvae	0–1%	>1%
Mean frequency of late dead larvae	0–2%	>2%
Mean frequency of growth-retarded larvae	0–4%	>4%
	Background response. The upper limit is the 90th percentile of response at reference sites	Elevated effect levels

Comparisons of datasets (Table 13.1) show that class II (i.e. elevated mean frequencies of malformed larvae and late dead larvae) mainly have been found in areas that are not regarded as reference sites (i.e. suspected to be more polluted) (Figure 13.3). However, only one of the datasets shows significantly elevated levels of growth-retarded larvae in the broods.

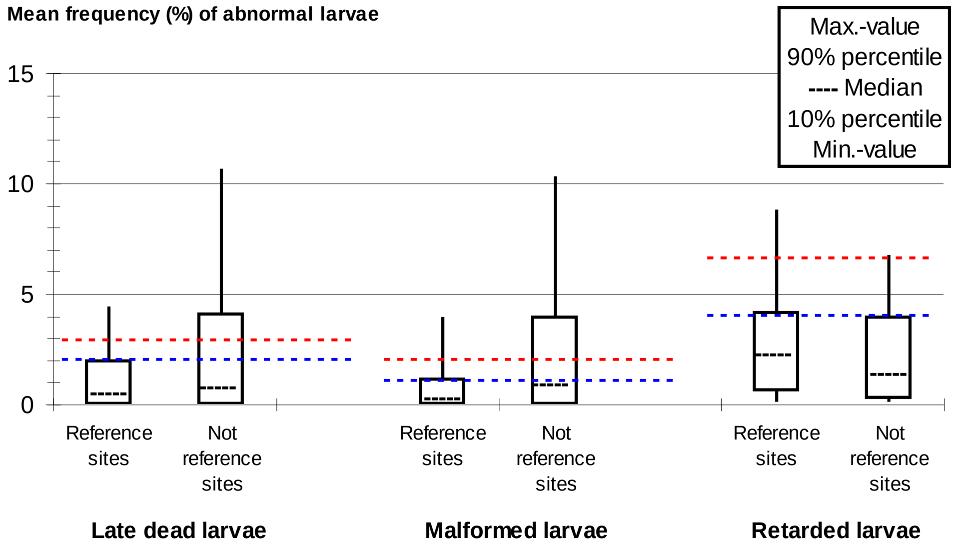


Figure 13.3. Comparison of data distribution of data on mean frequencies of late dead, malformed, and growth-retarded larvae in eelpout broods from reference sites and areas not regarded as reference sites. The blue dotted line refers to the 90th percentile of data from the reference sites. The red dotted line refers to significantly elevated levels compared with the 90th percentile of the reference sites.

13.2.2 Assessment criteria related to individual broods with >5% abnormal larvae

Some Swedish and Danish eelpout studies from the Baltic Sea, the Kattegat, and the Skagerrak have also used the frequency of pregnant eelpout containing elevated proportions of late dead or malformed larvae in the broods (e.g. >5%) as a measure of impaired reproduction in eelpout.

In areas which were considered as reference sites, only low frequencies have been found, if any (90th percentiles: 5%), of the pregnant eelpout containing elevated frequencies of late dead and malformed larvae in the broods (i.e. >5%).

Table 13.2. Proposed assessment criteria for the frequencies of pregnant eelpouts, which contain >5% malformed larvae and late dead larvae in their broods

Assessment class	Class I	Class II
	Background response	
Frequency of broods with >5% malformed larvae	0–5%	> 5%
Frequency of broods with >5% late dead larvae	0–5%	> 5%
	Background response. The upper limit is the 90th percentile of response at reference sites	Elevated effect levels

Comparisons of the datasets show that class II (i.e. elevated frequencies of broods containing >5% late dead larvae and malformed larvae) can be found in several areas which are not regarded as reference sites (Figure 13.4).

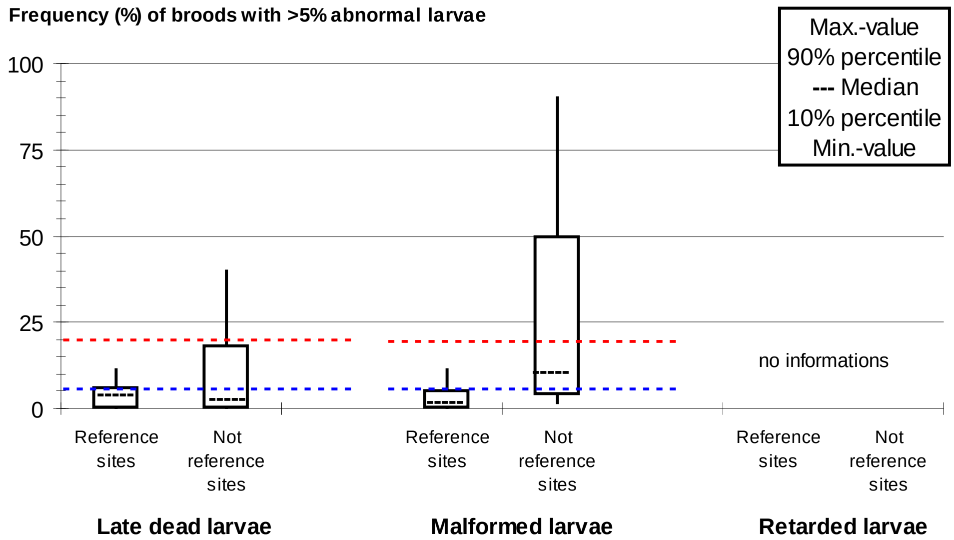


Figure 13.4. Comparison of data distribution of data on frequencies of broods containing >5% late dead larvae and malformed larvae from reference sites and areas not regarded as reference sites. The blue dotted line refers to the 90th percentile of data from the reference sites. The red dotted line refers to significantly elevated levels compared with the 90th percentile of the reference sites.

The assessment criteria including the existing data and the statistical analyses will be reviewed as part of a BONUS+ project, called BALCOFISH.

13.3 Conclusions

The use of reproductive success of eelpout with focus on the occurrence of abnormal developed embryo and larvae in the broods seems to be a potential tool for assessing environmental impact on fish reproduction, because differences have been shown between areas regarded as reference sites and not.

Proposals for two assessment classes of effect levels (I and II) have been derived based on the 90th percentile of the datasets of mean frequencies as well as broods containing >5% of late dead larvae, malformed larvae, and growth-retarded larvae, respectively.

These assessment criteria seem especially useful for the data consisting of occurrences of late dead larvae and malformed larvae, where significantly elevated levels can be found in several areas not regarded as reference sites, whereas the occurrence of growth-retarded larvae may be less useful.

14 Background document: metallothionein (MT) in blue mussels (*Mytilus edulis*, *M. galloprovincialis*)

Ketil Hylland

14.1 Introduction

Metallothionein (MT) is a low-molecular-weight, cysteine-rich protein, metal-binding protein found in all vertebrates and most invertebrates. The natural functions of different isoforms of the protein are under discussion and probably vary between species and for tissues within a species. Most forms are involved in metal-sequestration, thereby possibly:

- regulating cellular processes requiring Zn and/or Cu; and
- binding and thus temporarily detoxifying non-essential elements such as Cd and Hg.

In addition, MT has been suggested to be involved in the cellular defence against free radicals (mainly owing to the large number of sulfhydryl groups). Most of the data available are for liver or hepatopancreas, but there are also some data for gills in both fish and mussels.

In marine fish species, MT concentration in tissues has been found to be most strongly associated with Zn and Cu levels, although Cd may also result in minor increases in areas with metal stress (Hylland *et al.*, 2009). Because tissue requirements, and hence concentrations, of essential elements such as Zn and Cu will also be affected by exposure to other contaminants, interpretation of MT in fish as a simple biomarker for metal stress has not been straightforward except in areas with exceptionally high metal levels (predominantly freshwater).

MT in marine invertebrates, particularly mussels, was reviewed recently (Amiard *et al.*, 2006). Two main forms of the protein have been identified in blue mussel species, MT-10 and MT-20 (the names reflecting their approximate molecular size). There are a number of genes encoding MT-10 and fewer encoding MT-20 mRNA in *Mytilus edulis* and *M. galloprovincialis* (reviewed in Aceto *et al.*, 2011). Gene transcripts of MT-10 and MT-10 intronless genes are orders of magnitude higher than MT-20 under normal metabolism, but the relative increase in MT-20 gene expression under conditions of metal stress is very much higher than that of MT-10 isoforms (Aceto *et al.*, 2011).

Three main protocols have been used to quantify metallothionein in mussel tissues:

(i) the electrochemical differential pulse polarography method (DPP; Olafson and Thompson, 1974);

(ii) metal substitution; and

(iii) the spectrophotometric sulfhydryl method (Viarengo *et al.*, 1997).

In addition, an immunochemical assay has been described, but this has not been used to any extent (Roesijadi *et al.*, 1988). The three former methods rely on the content of sulfhydryl groups (SH-groups) in MT and its small size. There has been an international intercalibration of method (iii) through MED POL (Viarengo *et al.*, 2000) and of fish MT using all three methods within the BEQUALM framework (K. Hylland, pers. comm.). Unfortunately, the three methods do not yield the same values when applied to identical samples. Method (i) appears to provide the most reliable values and is the method that has been most extensively validated; method

(ii) is sensitive to the affinity of different metals for MT. Cu bound to MT under normal conditions has high affinity and must either be replaced by a metal with even higher affinity (e.g. Ag or Hg) or displaced prior to incubation with, for example, Cd. Method (iii) gives different results to the other methods, resulting in either over- or underestimation. None of the methods are able to distinguish between MT-10 and MT-20.

Although MT isoforms are thought to be predominantly cytosolic, they have been shown to be present in the nucleus in blue mussels, presumably as part of a regulatory function (Castillo and Robinson, 2008). The quantification methods currently used will mainly include cytosolic MT (nuclei will be excluded in the first separation of the work-up process), but this is not thought to be problematic as the total amount in the cell will be dominated by MT present in the cytosol.

An increasing number of studies have quantified mRNA for MT-10 and/or MT-20 (Dondero *et al*., 2005). There appears to be a large increase in MT-20 following metal stress under controlled experimental conditions, whereas increases in MT-10 are less dramatic (Zorita *et al*., 2007a). Similar results have been found in field studies (Aceto *et al*., 2011). MT-20 appears to be more resistant to oxidative stress than does MT-10 (Vergani *et al*., 2007). mRNA is a much more transient response than protein levels, however (as measured by the methods presented above), and there is a need for more knowledge of response dynamics prior to applying the method in a monitoring context.

MT in tissues is most commonly expressed on either a wet-weight or dry-weight basis (back calculated), but some authors also express it on the basis of cytosolic protein (the common standard for fish MT). Appropriate factors can be applied to convert from one basis to another, albeit introducing some error.

14.2 Concentrations in reference areas

A range of studies have quantified MT using DPP in whole mussels, hepatopancreas, and/or gill in *M. edulis* (Table 14.1) or *M. galloprovincialis* (Table 14.2). A smaller number of studies have been using the sulfhydryl method (Table 14.3). Early analyses using metal-substitution assays will have underestimated MT and have not been included in this overview.

Table 14.1. Mean concentrations of MT in different tissues of *Mytilus edulis*; expanded from Amiard *et al.* (2006). Some values were read off figures. Values reported on a dry-weight basis were recalculated to wet weight using a factor of 0.8 (water content; see e.g. Williams, 1970) and from protein-standardized values using a factor of 0.08 (assuming 2/3 cytosolic protein and a protein content of 60% of dry wt; Dare and Edwards, 1975)

Tissue	Original value	Factor	MT (µg g⁻¹ ww)	Reference
Whole animal	2.43	0.2	0.49	Bebianno and
	2.75	0.2	0.55	Langston (1989)
	0.55	1	0.55	Bebianno and
	0.55	1	0.55	Langston (1991)
	0.35	1	0.35	Amiard-Triquet *et al.* (1998)
				Amiard *et al.* (2008)
				Amiard *et al.* (2008)
Digestive gland	2.25	1	2.25	Amiard *et al.* (1998)
	8.04	0.2	1.61	Bebianno and
	8	0.2	1.6	Langston (1989)
	8.8	0.2	1.76	Bebianno and
	1.8	1	1.8	Langston (1991)
				Amiard-Triquet *et al.*

	1.6	1	1.6	(1998) Pellerin and Amiard (2009) Geffard *et al.* (2005)
Gills	0.3 2.2 1.7 8 0.23	1 0.2 0.2 0.08 1	0.3 0.44 0.34 0.63 0.23	Amiard *et al.* (1998) Bebianno and Langston (1991) Amiard-Triquet *et al.* (1998) Géret *et al.* (2002) Geffard *et al.* (2005)

Table 14.2. Mean concentrations of MT in different tissues of *Mytilus galloprovincialis*; expanded from Amiard *et al.* (2006). Some values were read off figures. Values reported on a dry-weight basis were recalculated to wet weight using a factor of 0.8 (water content; see e.g. Williams, 1970) and from protein-standardized values using a factor of 0.08 (assuming 2/3 cytosolic protein and a protein content of 60% of dry wt; Dare and Edwards, 1975)

Tissue	Original	Factor	MT (µg g^{-1} ww)	Reference
Whole animal	12.1 1.21 3.21 0.5	0.2 1 0.2 1	2.4 1.21 0.64 0.5	Bebianno and Machado (1997) Raspor *et al.* (1999) Bebianno and Langston (1992) Mourgaud *et al.* (2002)
Digestive gland	4.09 2.1 45	1 1 0.08	4.09 2.1 3.56	Raspor *et al.* (1999) Pavicic *et al.* (1993) Zorita *et al.* (2007b)
Gills	0.62 2.35	1 0.2	0.62 0.47	Raspor *et al.* (1999) Bebianno and Langston (1998)

Table 14.3. Mean concentrations of MT in different tissues of *Mytilus edulis* and *M. galloprovincialis*. Some values were read off figures. Values reported on a dry-weight basis were recalculated to wet weight using a factor of 0.8 (water content; see e.g. Williams, 1970) and from protein-standardized values using a factor of 0.08 (assuming 2/3 cytosolic protein and a protein content of 60% of dry wt; Dare and Edwards, 1975)

Tissue	Original	Factor	MT (µg g^{-1} ww)	Reference
M. edulis				
Whole animal	0.04	1	0.04	Brown *et al.* (2004)
Digestive gland	0.11 0.16	1 1	0.11 0.16	Da Ros *et al.* (2007) Schiedek *et al.* (2006)
M. galloprovincialis				
Whole animal	20 0.45	0.08 1	1.6 0.45	Funes *et al.* (2006) Domouhtsidou *et al.* (2004)
Digestive gland	0.3 0.45 0.15	1 1 1	0.3 0.45 0.15	Viarengo *et al.* (2000) Domouhtsidou *et al.* (2004) Donnini *et al.* (2007)
Gills	40	1	40	Hamer *et al.* (2008)

14.3 Confounding factors

Some studies indicate seasonal variation in MT in mussels with large changes during the spawning period and lower concentrations of the protein, but more stable values in the rest of the year (Raspor *et al.*, 2004; Geffard *et al.*, 2005; Zorita *et al.*, 2007b). However, other studies have found higher values in autumn (Pellerin and Amiard, 2009). This may be the result of different periods of spawning and/or species differences; *M. galloprovincialis* was used in the Mediterranean and *M. edulis* on the French Atlantic coast. A recent study has indicated that *M. galloprovincialis* dominates the Mediterranean / Iberian peninsula and *M. edulis* the French coast, but that there are mixed populations of the two and *M. trossulus* in some areas of northern Europe (Kijewski *et al.*, 2011).

All available data clearly show that there is a strong seasonal dynamic in tissue metal concentration and metallothionein in blue mussels. There appear to be differences between the two species, possibly associated with different spawning periods.

14.4 Assessment criteria

The medians or averages from different studies with the three tissues were remarkably similar for *M. edulis*; provisional background assessment criteria (BAC) were constructed using the 90th percentile of averages/medians from the literature: whole body, 0.6 μg g^{-1} ww; digestive gland, 2.0 μg g^{-1} ww; and gills, 0.6 μg g^{-1} ww. These values comprise medians for a full seasonal cycle.

BAC for *M. galloprovincialis* generated in a similar way were: whole body, 2.0 μg g^{-1} ww; digestive gland, 3.9 μg g^{-1} ww; and gills, 0.6 μg g^{-1} ww. As above, the values are medians for a seasonal cycle.

MT concentrations measured using the sulfhydryl method produced results very different from those found using DPP; no assessment criteria have been established for this method.

15 Background document: histopathology of mussels (*Mytilus* spp.) for health assessment in biological effects monitoring

John Bignell, Miren P. Cajaraville, and Ionan Mariogómez

15.1 Background

Mussels have long been used for the measurement of pollutants and the biological effects of contaminants in the aquatic environment (Bayne, 1976; Goldberg *et al.*, 1978; Widdows and Donkin, 1992; Granmo, 1995; Salazar and Salazar, 1995). They are widespread, sessile, possess the ability to accumulate chemicals, and exhibit a wide range of biological responses. They are able to tolerate wide-ranging salinity conditions and are also seen attached to piers and gravelly substrates. This makes them well placed as a sentinel species in programmes designed to monitor the marine environment. Over the years, numerous studies using mussels have demonstrated the impact of anthropogenic inputs into the aquatic environment. Early studies such as the "Mussel Watch" programme (Goldberg *et al.*, 1978) were primarily designed to evaluate pollution within coastal waters by measuring levels of pollutants within tissues of mussels (and other bivalves). In comparison, relatively few studies focused on the effect of these chemicals on their test organisms.

Over the years, there has been increased emphasis placed on integrated assessments in national and international monitoring programmes within the Oslo–Paris Commission (OSPAR) region that incorporate both chemical analyses and their biological effects. A range of contaminants exist within the aquatic environment, which may elicit an assortment of biological responses. As such, it is well established that integrated techniques provide a more robust approach for the overall health assessment of aquatic organisms and their environment than the application of a single technique in isolation.

Histopathology (of aquatic organisms) is a valuable tool for assessing the health of individuals and populations because it incorporates measures of reproductive and metabolic condition and allows for the detection of a range of pathogens that may affect morbidity and mortality. In addition to its role as a "baseline" measure of health, histopathology has been used to investigate changes related to polycyclic aromatic hydrocarbon (PAH), polychlorinated biphenyl (PCB), and heavy metal exposure in mussels (Sunila, 1984; Lowe and Pipe, 1987; Auffret, 1988; Kluytmans *et al.*, 1988; Marigómez *et al.*, 2006). Mussel histopathology has been designated a promising technique (tissue response) for inclusion within the "mussel integrated approach". It provides an effective set of tools for the detection and characterization of toxicopathic pathologies, which are increasingly being used, in addition to disease, as indicators of environmental stress.

Histopathology is also complementary to other techniques used to monitor the biological effects of contaminants as it can help to dissociate markers of underlying health or disease condition from those associated with exposure to contaminants. The advent of genomic and post-genomic technologies increases the potential utility of histopathology in quality assurance and quality control of sample groups for analysis (e.g. by selecting homogeneous group attributes and to control for potential variation among individuals). This approach should help to reduce uncertainties associated with the potential confounding effects of pathogens when trying to identify the specific effects of toxicant exposure on host gene, protein, and metabolite profiles (Stentiford *et al.*, 2005; Ward *et al.*, 2006; Hines *et al.*, 2007). In this respect, it can be considered as a means to provide supporting information for measures (biomarkers) that specifically aim to assess historic exposure to, or effect of, a contaminant. Histopathology, therefore, provides a ”phenotypic anchor” against which this specific data can be assessed (Stentiford *et al.*, 2005).

This report provide a description of numerous health parameters that can be employed in monitoring programmes designed to assess the biological effects of contaminants. It also describes pathology that has been previously associated with contaminant exposure, but may also result from exposure to pathogens. Although the latter may initially seem misplaced in this report describing contaminant-induced pathology, it is important to note that disease conditions of pathogenic aetiology can result in pathology that may appear contaminant-related to the untrained eye. Therefore, it is essential for an individual to possess the ability to be able to distinguish between contaminant- and pathogen-related pathology.

15.2 Sampling and dissection for formalin-fixed, paraffin-embedded (FFPE) histology

When sampling mussels in the field, mussels should be carefully removed from their substrate by cutting the byssus threads with a pair of scissors. This will help to reduce stress that may act as a confounding factor when integrating with other sensitive biological effects techniques, such as the neutral red retention (NRR) assay. Mussels should be placed in a suitable insulating container and kept cool and moist during prompt transport back to the laboratory. This can be achieved by using a combination of ice packs, wet paper towels, and/or seaweed.

With integrated studies becoming more widespread, adopting a quality assurance approach is considered an important practice. So that potential post-sampling artefacts are minimized, mussels should be processed as soon as possible following removal from water. When dealing with samples distributed over a large geographical area (e.g. from national/international monitoring programmes), it may not always be possible to process samples immediately or relatively soon after. This is primarily because samples require lengthy transit to the laboratory, thus delaying subsequent processing. Under these circumstances, efforts should be made to keep the time from sampling until the time of processing equivalent in duration between all samples. Currently, the number of individual mussels required for histology is 50, although this will be reviewed in the preparation of an ICES *TIMES* series document for mussel histopathology.

The dissection process is an extremely important stage in the histological process, and it is crucial that it is conducted in a standardized manner. Standardized dissections ensure greater comparability between samples and simplify downstream histological analysis. It is essential to achieve good-quality cross sections that are not too thick to ensure adequate penetration of tissues by the fixative. Mussels should be treated with care so as not to cause any damage to any of the tissues. Any damage to tissues during dissection may prevent good-quality cross sections being obtained.

In order to gain access to the visceral mass within the shell, hold the mussel with the posterior shell edge on a suitable work surface such as a dissection board. Insert a scalpel blade into the midventral byssal cavity (do not insert too far as this will damage tissues situated along the dorsal shell edge) followed by a downward movement resulting in the cutting of the posterior adductor muscle. Carefully open the two shell halves to reveal the visceral mass. Using a scalpel or scissors, remove any byssus threads that may hinder any microtomy carried out at a later stage. Do not remove byssus threads by pulling (threading) as this may cause undue stress to the mussel. Starting with one shell half at first, carefully separate the mantle tissue from the inner shell surface using the flat edge of a scalpel blade. Care should be taken not to "slice" the mantle with the scalpel blade itself. To an untrained individual, this can be challenging at first; however, it is soon overcome. The most successful approach is to combine the use of "teasing" and "scraping". Brush aside the partially removed visceral mass into the remaining shell half and sever the posterior retractor muscles. Once complete, the empty shell half can be removed from the remaining half by disassociation of the shell ligament (a simple twist of the empty shell will suffice). In a similar manner to the above, the mantle tissue should be teased away from the inner shell surface of the remaining shell half. This process can be made easier by resting the previously dissected tissue onto a work surface while working with the remaining tissue. Once complete, the entire visceral mass should be removed from the remaining shell and placed onto a dissection board. Using a razor blade or scalpel, a slightly angled 3-mm slice across the ventral and posterior axis should be obtained towards the anterior end of the visceral mass. This will ensure that the main organs of interest (gonad, gills, mantle, digestive gland, kidney, foot) are incorporated into a single standardized section. Using forceps, carefully transfer the cross section into a histo-cassette before placing into Davidson's Seawater Fixative or suitable alternative. The use of histo-cassettes is highly recommended because of their ability to ensure that the cross section remains intact during the fixation process. Allow fixation to proceed for a minimum of 24 hours, with periodic agitation throughout. The use of a "rocker" will make this easier.

15.3 Sampling and dissection for histochemistry

Histochemical techniques on frozen tissue sections (obtained by cryotomy) are needed in order to evaluate lysosomal alterations described below. As such, further dissection is required when incorporating these techniques.

For cryotomy, a small cube of digestive gland should be dissected from a minimum of 10 individual mussels and snap-frozen onto a cryotome chuck in two rows of five, using a suitable cryo-embedding compound such as OCT. Snap-freezing can be achieved using liquid nitrogen or a commercially available cryobath. For better integration of data, it is possible to obtain frozen samples from the same mussels identified for FFPE histology. Chucks should be transported deep-frozen to the laboratory and subsequently stored at –80°C.

15.4 Histology

FFPE histology is the most widely used histological process; however, resin-based embedding techniques can also be employed. For FFPE histology, tissues are dehydrated through a series of graded alcohols, followed by clearing and embedding within paraffin wax. Finally, tissues are placed into moulds containing molten wax that are subsequently cooled to produce a rigid support medium (block) for microtomy. Detailed protocols will be included in an ICES TIMES series document on mussel histopathology.

Using a microtome, the face of the tissue blocks are "trimmed" or "faced" in order to expose the maximum surface area of the mussel embedded within the block. Occasionally, sand or residual byssus may be encountered during sectioning, which may prevent suitable sections being obtained. Under these circumstances, it may be possible to remove these artefacts from the block face using a small sharp implement such as a pin or needle. Care should be taken not to cause any unnecessary damage to the surrounding tissues. This ensures that all areas of interest are included during sectioning. Tissue sections are obtained at 3–5 μm and floated onto a pre-heated water bath (35–40°C) containing a suitable tissue adhesive (e.g. Sta-On, Surgipath, UK). Alternatively, commercially available slides that have been pretreated with saline or electrostatically charged can be used. Sections are adhered to a glass microscope slide by inserting the slide vertically into the water bath adjacent to the floating section and lifting straight up. Following sectioning, slides should be dried overnight on a suitable hotplate. Alternatively, a section dryer can be used which can decrease the time taken for slides to dry. Whatever drying method is employed, it is important to ensure that all moisture has been removed from slides prior to staining. Subsequently, sections are stained with haematoxylin and eosin or a suitable alternative. Following staining, the result should resemble Figure 15.1. This approach produces a uniform histological section that (a) incorporates all of the target organs of interest and (b) makes for a simpler microscopic examination owing to the standardized orientation of the tissues and organs. Using a low-magnification objective, the histopathologist should scan the slide for any abnormalities before further examination at higher magnifications. It is recommended to observe slides "blind" (i.e. without prior knowledge to geographical location or exposure groups), in order to reduce bias that may otherwise be introduced to the interpretation.

Detailed sampling procedures are outlined in the *ICES TIMES* document.

Figure 15.1. Representative overview of standard haematoxylin and eosin histological mussel section.

15.5 Quality assurance

At present, there is no quality assurance scheme in place for mussel histopathology. It is envisaged that this will be run in a similar manner to the BEQUALM Fish Disease Programme currently organized by Cefas.

15.6 Health parameter measurements

The following parameters can be measured quantitatively or semi-quantitatively with histological techniques, cell-type composition in digestive gland epithelium, digestive tubule epithelial atrophy and thinning, lysosomal alterations and inflammation and are described in detail below.

15.7 Cell-type composition in digestive gland epithelium

Under normal physiological conditions, the digestive cells outnumber basophilic cells, but under different stress situations, including exposure to pollutants, the relative occurrence of basophilic cells is apparently augmented (Rasmussen *et al.*, 1985; Lowe and Clarke, 1989; Cajaraville *et al.*, 1990, Marigómez *et al.*, 1990, 1998; 2006; Zorita *et al.*, 2006, 2007b; Garmendia *et al.*, 2011a). Changes in cell-type composition in the digestive gland epithelium constitute a common response in molluscs that may lead to disturbances in food digestion and xenobiotic metabolism and accumulation (Marigómez *et al.*, 1998). These changes have been attributed to basophilic cell proliferation (Cajaraville *et al.*, 1989; Lowe and Clarke, 1989; Marigómez *et al.*, 1990), but it has been recently concluded that it mainly results from digestive cell loss and basophilic cell hypertrophy (Zaldibar *et al.*, 2007), which is a fast, inducible, and reversible response that can be measured in terms of volume density of basophilic cells (VvBAS). In clean localities and in experimental control conditions, VvBAS is usually below 0.1 $\mu m^3\ \mu m^{-3}$, but after exposure to pollutants, VvBAS may surpass 0.12 $\mu m^3\ \mu m^{-3}$ (Marigómez *et al.*, 2006).

A stereological procedure is applied in order to quantify the VvBAS as a measure of digestive cell loss by counting on haematoxylin and eosin-stained, digestive gland paraffin sections (Soto *et al.*, 2002). Cell counts (digestive and basophilic cells) are made in one field randomly selected per mussel ($n = 10$) to complete a total of ten counts per experimental group, with the aid of a drawing tube attached to a light microscope using a 20× objective lens. A Weibel graticule (multipurpose test system M-168) is used, and hits on basophilic cells and on remaining digestive epithelium are recorded to calculate VvBAS according to the Delesse's principle:

$$\text{VvBAS } (\mu m^3\ \mu m^{-3}) = x/(m + x);$$

where x is the number of hits on basophilic cells and m is the number of hits on digestive cells. The statistical significance of changes in VvBAS volume is determined according to parametric tests (e.g. ANOVA, Duncan's test for comparison between pairs of means; $p < 0.05$). Assessment criteria should be considered as:

Background:	$<0.12\ \mu m^3\ \mu m^{-3}$
Elevated:	$0.12–0.18\ \mu m^3\ \mu m^{-3}$
High:	$>0.18\ \mu m^3\ \mu m^{-3}$

15.8 Digestive tubule epithelial atrophy and thinning

The best documented cellular alteration in bivalves is apparent atrophy or "thinning" of the digestive gland epithelium. The digestive gland of mussels is greatly dynamic and plastic. The morphology of digestive alveoli undergoes severe changes even during normal physiological processes (i.e. trough every digestion cycle; Langton, 1975). Changes in the normal phasic activity may be attributed to environmental factors, such as food availability or saline and thermal stress (Winstead, 1995) as well as exposure to pollutants. In particular, it has been widely demonstrated that molluscs exposed to pollutants exhibit a net mass loss in the digestive gland epithelium that gives rise to abnormal epithelial thinning and finally atrophy (Lowe *et al*., 1981; Couch, 1984; Lowe and Clarke, 1989; Vega *et al*., 1989; Cajaraville *et al*., 1992; Marigómez *et al*., 1993; Garmendia *et al*., 2011a). Atrophy and epithelial thinning constitute a non-specific, fast, inducible, and slowly or not recoverable response to stressful environmental conditions that can be measured after semi-quantitative scoring (Table 15.1; Kim *et al*., 2006) or after quantitative morphological analysis in terms of MPTW (mean proportion of tubule width; Robinson, 1983); or in terms of mean epithelial thickness (MET) and the relative parameters mlR/MET and MET/MDR (Lowe *et al*., 1981; Vega *et al*., 1989; Cajaraville *et al*., 1992; Marigómez *et al*., 1993, 2006; Garmendia *et al*., 2011a), where mlR is the mean luminal radius and MDR the mean diverticular radius. mlR/MET ratio is more sensitive than MET alone. The alterations in these parameters are used as tissue-level biomarkers in ecosystem health assessment (Garmendia *et al*., 2011a).

Table 15.1. Description of a semi-quantitative scoring index for digestive tubule epithelial atrophy and thinning

Stage	Response	Description
0	None	Normal tubule thickness (0% atrophy). Lumen nearly occluded, few tubules exhibiting slight atrophy
1	Low	Epithelium averaging less than one-half (50%) normal thickness (stage 0), most tubules show some atrophy although some tubules appear normal
2	Elevated	Epithelium averaging ca. 50% of normal thickness (stage 0)
3	High	Epithelium thickness greater than one-half (50%) atrophied, most tubules affected, some tubules extremely thin (fully atrophied)
4	Severe	Epithelium extremely thin (100% atrophied), nearly all tubules affected

Adapted from Ellis *et al*. (1998).

Most commonly, a planimetric procedure has been applied to quantify changes in size and shape of the digestive alveoli (Vega *et al*., 1989), resulting in apparent epithelial thinning. In all, 50–100 tubular profiles per sample (two profiles per field in five fields per mussel in 5–10 mussels per sample) are recorded in an image-analysis system attached to a light microscope using a 20× objective lens. The five measurement fields are selected at given intervals throughout the tissue section, the direction of movement always following a zigzag pattern. Alternatively, tubular profiles can be drawn with the aid of a drawing tube attachment to the light microscope and then digitized for data input to a computer. Other methods are also available since the final goal is just calculating the section areas of the lumen and the whole tubule profile, which can be done by image-analysis systems (after data input into the computer), by hand (e.g. using millimetre paper), or by point counting onto a Weibel stereological graticle (Weibel, 1979). MET, mlR, and MDR are quantified (in μm) and the ratios mlR/MET and MET/MDR (in μm μm^{-1}) are calculated as integrative measures of changes in the alveolar morphology, epithelial thinning included, as follows:

$$\text{MET} = 2\,(A_o - A_i) / (P_o + P_i);$$
$$\text{MLR} = \sqrt{}(A_i / \pi); \text{ and}$$
$$\text{MDR} = \sqrt{}(A_o / \pi);$$

where A_o is the section area of the whole tubule profile, P_o is the perimeter of a circle with area A_o, A_i is the section area of the lumen profile, and P_i is the perimeter of the corresponding circle with area A_i. The statistical significance of changes in these parameters is determined according to parametric tests (e.g. ANOVA, Duncan's test for comparison between pairs of means; $p < 0.05$). mlR/MET values between 0.7 μm μm^{-1} (spring–summer) and 1.2 μm μm^{-1} (winter) have been recorded in *M. galloprovincialis* of reference localities in southern Bay of Biscay, whereas after exposure to pollutants or stress in long-term laboratory manipulation, mlR/MET surpasses 1.6 µm µm^{-1} (Marigómez *et al*., 2006).

15.9 Lysosomal alterations

Lysosomal responses are widely used as effect biomarkers indicative of the general stress provoked by pollution in the marine environment. Lysosomes are cell organelles containing acid hydrolases. The digestive cells of mussels possess a complex endolysosomal system that is primarily involved in the uptake and digestion of food materials as well as in processes of pollutant accumulation and detoxification. Endolysosomes and heterolysosomes occupy the majority of digestive cell cytoplasm and are reactive for marker hydrolases such as *N*-acetyl hexosaminidase, β-glucuronidase, and acid phosphatase (Izagirre *et al.*, 2009; Izagirre and Marigómez, 2009). Lysosomal responses to environmental stress fall into essentially three categories: increased lysosomal size, reduced membrane stability, and changes in lysosomal contents (Marigómez and Baybay-Villacorta, 2003).

15.9.1 Lysosomal enlargement

Diverse sources of environmental stress (chemical pollution, salinity changes, elevated temperature, malnutrition, reproductive stress) are known to provoke an increase in the size of digestive cell lysosomes in mussels, often accompanied by increased enzyme activity and lysosome numbers, which may compromise intracellular digestion and detoxification capacity (Moore, 1985, 1988; Lowe, 1988; Cajaraville *et al*., 1989, 1995; Marigómez *et al*., 2005, 2006; Domouhtsidou and Dimitriadis, 2001; Garmendia *et al*., 2011b). These lysosomal structural changes (LSC) have been commonly determined by image analysis of digestive gland cryotome sections where β-glucuronidase is employed as lysosomal marker enzyme. The final calculations of the structural parameters are, in most cases, based on the equations published by Lowe *et al*. (1981). The structural parameters are lysosomal volume density (*Vv*), surface density (*Sv*), surface-to-volume ratio (*S*/*V*), and numerical density (*Nv*). Although the four stereological parameters together provide complete information about the size, size-class distribution, and number of lysosomes in mussel digestive cells, *Vv* can be sufficient to detect changes in the size of the endolysosomal system and is, therefore, the most used parameter.

15.9.2 Stereological determination of lysosomal enlargement

The histochemical reaction for β-Gus is demonstrated, as in Moore (1976), with the modifications described by Cajaraville *et al*. (1989). Slides are kept at 4°C for 30 min and then at room temperature for 5 min prior to staining. Sections (8 µm) are incubated in freshly prepared β-Gus substrate incubation medium consisting of 28 mg naphthol AS-BI-β-glucuronide (Sigma, N1875) dissolved in 1.2 ml 50 mM sodium bicarbonate, made up to 100 ml with 0.1 M acetate buffer (pH 4.5) containing 2.5% NaCl and 15% polyvinyl alcohol, for 40 min at 37°C in a shaking water bath. After incubation, slides are rinsed in a 2.5% NaCl solution for 2 min at 37°C in a shaking water bath and then transferred to a postcoupling medium containing 0.1 g Fast garnet GBC (Sigma, F8716) dissolved in 100 ml 0.1 M phosphate buffer (pH 7.4 containing 2.5% NaCl) for 10 min in the dark and at room temperature. Thereafter, the sections are fixed for 10 min at 4°C in Baker's formol calcium containing 2.5% NaCl and rinsed briefly in distilled water. Finally, sections are counterstained with 0.1% Fast green FCF (Sigma, F7252) for 2 min, rinsed several times in distilled water, mounted in Kaiser's glycerine gelatine, and sealed with nail varnish. Then, *de visu* grading and scoring can be applied to grossly determine the extent of lysosomal enlargement (Lowe, 1988), which can be straightforward and very useful in cases of extreme symptoms. However, quantifying lysosomal enlargement by hand stereology (Cajaraville *et al*., 1989, 1992) or by image analysis (Marigómez *et al*., 2005; Izagirre and Marigómez, 2009) can provide evidence of more subtle lysosomal responses. Slides are viewed under a light microscope fitted with a 100× objective lens. A Weibel graticule (multipurpose test system M-168) is used, and hits on digestive cell lysosomes and on digestive cell cytoplasm are recorded to calculate *VvLYS*, *SvLYS*, *S/VLYS*, and *Nv LYS* according to Lowe *et al*. (1981). Five measurements are made per section in each of the 5–10 individuals per sample. The stereological formulae include a correction factor for particles with an average diameter smaller than the section thickness (Lowe *et al*., 1981). For this reason the average diameter of at least 90 lysosomes must be directly measured at the light microscope with the aid of a graded eyepiece or similar device (or directly by the image-analysis system):

$VvLYS\ (\mu m^3\ \mu m^{-3}) = K \times AA;$
$SvLYS\ (\mu m^2\ \mu m^{-3}) = (4/t) \times AA;$
$S/VLYS\ (\mu m^{-1}) = 4/(t \times K);$ and
$NvLYS\ (\mu m^2\ \mu m^{-3}) = (4 \times AA \times n)/(t \times \pi \times \Sigma\ Y_{i2})$

where $AA = x/m$ and $K = [2/(3 \times t)]\ (\Sigma Y_{i3} / \Sigma Y_{i2})$; and where x is the number of hits on digestive cell lysosomes, m is the number of hits on digestive cells (lysosomes included), t is the section thickness (i.e. 8 µm), n is the number of lysosomes whose diameter has been measured, and Y are lysosomal diameters (Y_1, Y_2, … Y_{90} for $n = 90$).

Lysosomal structural changes test parameters can be tested using analysis of variance. *VvLYS* and *NvLYS* data may need to be logarithmically transformed previous to the statistical analyses because the variance within individuals may depend on the mean. Parametric tests for multiple comparisons between paired means (e.g. Duncan's test) can be further applied to detect significant ($p < 0.05$) differences between means.

In general terms, lysosomes become enlarged under stress conditions, which are reflected as increases in *VvLYS* and *SvLYS* values, concomitant with lowered *S/VLYS* values (Cajaraville *et al*., 1995; Marigómez *et al*., 2005). In certain cases, lysosomal enlargement is accompanied by increased *NvLYS*, (more lysosomes relative to digestive cell cytoplasm), but reductions in *NvLYS* have also been reported. On the other hand, exposure to pollutants may also elicit an intricate response that includes different phases (Marigómez and Baybay-Villacorta, 2003): (a) transient lysosomal enlargement; (b) transient lysosomal size reduction; and finally (c) lysosomal enlargement after long-term exposure. Overall, reference values for these lysosomal parameters vary with season, but $VvLYS > 0.002\ \mu m^3\ \mu m^{-3}$ and $S/VLYS > 5$ may be indicative of the existence of a degraded health status in mussels that correlates with, for example, the degree of exposure to pollutants.

15.10 Inflammation

Inflammation affects all tissues and organs and is particularly obvious in mussels that have been adversely affected by contaminants (Couch, 1985; Auffret, 1988). Although this may be true, it is important to remember that the presence of pathogens can also result in a host immune response (but not always) manifested as inflammation. Inflammation is observed as either diffuse, focal, or both in appearance throughout the vesicular connective tissue and at varying degrees of severity.

Haemocytic infiltration is generally characterized by the infiltration of granulocytes possessing an eosinophilic cytoplasm into the connective tissues. Care should be taken not to confuse this with normal circulating haemocytes that are often situated around the stomach and intestine. Heavy diffuse inflammation will appear as a marked increase in the number of circulating haemocytes situated throughout the majority of connective tissues and in between organs, such as the digestive diverticula and gonad. Haemocytic infiltration of the visceral mass in bivalves is generally considered to be indicative of stress, unrecognized injury, or submicroscopic agents in bivalves. Haemocytic infiltration could be interpreted as a repair process following tissue damage, although pathological effects could be exerted through acting as space-occupying lesions. Its presence has been suggested as a qualitative or quantitative index of stress, indicative of a loss of condition. Previous studies have reported haemocytic infiltration in response to starvation and spawning stress, shell damage, and exposure to pollutants.

Brown cell aggregates (foci) are generally small and possess varying quantities of the pigment lipofuscin and are often seen in elevated numbers in mussels from contaminated environments. Composed of serous cells, these phagocytes are mostly found within the connective tissue and possess the ability to physically remove endocytosed matter across epithelia via diapedesis. These cells are responsible for the metabolism of metal ions and can be found within the gills, which is an important organ for metal ion exchange (Marigómez *et al*., 2002). The occurrence of brown cell aggregates (foci) has been considered an indicator of stress caused by xenobiotics, as well as with age and reproductive stress. Brown cell aggregates are also observed within the gonad follicles following spawning, which is a normal event.

Large foci of inflammation, termed granulocytomas (composed of granulocytes), have previously been seen in mussels of both laboratory and field studies designed to monitor the effects of contaminants (see Table 15.2). Granulocytomas represent an inflammatory response to an irritant or pollutant, resulting in vascular occlusions. They are believed to result from chronic exposure to domestic and industrial waste products and have been reported in bivalves subjected to the impact of oil, chlorinated pesticides, and heavy metals. Granulocytomas are also associated with pathogens; therefore, it is important to look for any indication of infection in affected individuals. These lesions can be seen at varying degrees of severity from singular foci to large numbers affecting the majority of the connective tissues. Granulocytomas can vary largely in size. In mussels, the maximum size of a known parasitically induced granulocytoma is 400 μm; however, granulocytomas of unknown aetiology can be over 800 μm (up to 1500 μm).

Table 15.2. Description of a semi-quantitative scoring index for inflammation

Stage	Response	Description
0	None	No inflammatory foci can be seen within tissues. Brown cell foci rare
1	Low	Small numbers of inflammatory foci occupying ≤10% of the vesicular connective tissue (approximately 20 small foci) within standardized tissue cross section. Brown cell foci rare
2	Elevated	Increased numbers and/or size of inflammatory foci occupying between 10 and 50% of vesicular connective tissue. Foci may displace other structures. Areas of diffuse haemocyte infiltration may also be present. More brown cell foci predominantly within the vesicular connective tissue, stomach, and digestive gland epithelium
3	High	Significant inflammatory response—numerous and/or large inflammatory foci (possibly with granulocytoma present) occupying ≥75% of vesicular connective tissue. Widespread diffuse haemocytic infiltration may be present. More brown cell foci predominately within the vesicular connective tissue, stomach, and digestive gland epithelium. Increased pigment density

15.11 Assessment criteria

Several parameters have been identified as suitable for the development of assessment criteria. Other histological parameters can also be measured using histopathology, although many of these fluctuate, showing clear seasonal cycles (Bignell *et al.*, 2008). As such, the development of assessment criteria is not deemed appropriate. Nonetheless, the collection of these data can provide additional information on the health and physiology of the mussel. Parameters include reproductive markers such as adipogranular cells, gonadal apoptosis, atresia, hermaphroditism, and intersex. All health parameters will be described in a forthcoming *ICES TIMES* document.

The thresholds identified here (Table 15.3) have been determined using data collected as part of previous studies (Cajaraville *et al.*, 1992; Marigómez *et al.*, 2004, 2005, 2006; Bignell *et al.*, 2008). It must be emphasized that these thresholds are preliminary and will require further review as part of a holistic assessment of these histological parameters.

Table 15.3. Mussel histopathology preliminary thresholds determined from various studies

Biological effect	Qualifying comments	Background	Elevated	High
Mussel histopathology	VvBAS: Cell type composition of digestive gland epithelium (quantitative)	< 0.12 $\mu m^3\ \mu m^{-3}$	0.12–0.18 $\mu m^3\ \mu m^{-3}$	>0.18 $\mu m^3\ \mu m^{-3}$
	mIR/MET: Digestive tubule epithelial atrophy and thinning (quantitative)	< 0.7 $\mu m\ \mu m^{-1}$	1.2–1.6 $\mu m\ \mu m^{-1}$	>1.6 $\mu m\ \mu m^{-1}$
	VVLYS and *S/VLYS*: Lysosomal enlargement (quantitative)	*VvLYS* < 0.0002 $\mu m^3\ \mu m^{-3}$ *S/VLYS* > 4 $\mu m^2\ \mu m^{-3}$	0.0002–0.0004 $\mu m^3\ \mu m^{-3}$ *S/VLYS* < 4 $\mu m^2\ \mu m^{-3}$	*V*>0.0004 $\mu m^3\ \mu m^{-3}$ *S/VLYS* <<4 $\mu m^2\ \mu m^{-3}$
	Digestive tubule epithelial atrophy and thinning (semi-quantitative)	STAGE ≤1 (Mode)	STAGES 2–3 (Mode)	STAGE 4 (Mode)
	Inflammation (semi-quantitative)	STAGE ≤1 (Mode)	STAGE 2 (Mode)	STAGE 3 (Mode)

16 Background document: stress on stress (SoS) in bivalve molluscs

Concepción Martínez-Gómez and John Thain

16.1 Background

Contaminant exposure may alter the ability of organisms to survive environmental stress (Zwaan *et al.*, 1995; Viarengo *et al.*, 1995). Laboratory and field studies have demonstrated the applicability of anoxic/aerial survival as an early warning indicator of contaminant-induced stress. The effects of xenobiotics, including heavy metals, organometals, organics, and contaminated field sediments, on survival in air in invertebrates have been demonstrated (Zwaan and Eertman, 1996). Bivalve molluscs have been used in most studies, with marine mussels (*Mytilus* sp.) being the most common organism (Smaal *et al.*, 1991; Veldhuizen-Tsoerkan *et al.*, 1991; Eertman *et al.*, 1995; Viarengo *et al.*, 1995).

The reduction of survival in air, or stress on stress (SoS), is a simple, low–cost, whole-organism response and can show pollutant-induced alterations in an organism's physiology that render the animal more sensitive to further environmental changes. The method for determining SoS in mussels has been applied routinely to both toxicant-exposed mussels in laboratory studies and mussels collected in national monitoring programmes from polluted environments and along pollution gradients.

Laboratory studies have been conducted to establish relationships between toxicant concentrations in tissue and SoS. For example, it was demonstrated that short-term exposure to sublethal concentrations (less than micromolar) of pollutants such as Cu^{2+}, DMBA (9,10-dimethyl 1,2 benzanthracene), or Aroclor 1254 significantly reduced the capacity of mussels to survive in air. This effect was markedly dose-dependent, and was strongly increased by pollutant mixtures (Viarengo *et al.*, 1995). The accuracy of air exposure as a monitoring tool has been reported to reflect smaller differences between contaminant groups than other physiological measurements in mussels, such as byssal thread production rate (Moles and Hale, 2003). The measurement of survival in air also appeared to be a sensitive and statistically significant parameter for monitoring the effect of long-term exposure to crude oil (R. E. Thomas *et al.*, 1999).

16.2 Short description of methodology

Bivalve molluscs can survive for a long time in air, but individuals stressed by pre-exposure to pollutants show greater mortality than controls or individuals collected from a reference location. Both caged and native mussels can be used to assess the SoS response. The size of individuals for survival profiles must be selected from frequency distributions of the whole population under study. The individuals must be of a size approximating the mean shell length for the population. When mussels are collected from the intertidal zone, it is important to sample them when they are submerged (i.e. just before they are uncovered as the tide recedes or conversely when they are covered as the tide comes in).

For spatial/temporal studies, the same size range should be selected (ideally 4–5 cm). Forty mussels (four replicates of 10) are used for each determination of SoS. The mussels must be collected from the sampling site and immediately transported to the laboratory in insulated containers at a temperature of 5–10°C and in humid conditions (e.g. with damp paper or seaweed). Information that must be recorded includes: total number of animals sampled, date and time of sampling, sampling location and position (e.g. latitude and longitude), and seawater temperature.

Upon arrival at the laboratory, the 40 mussels are selected, placed on filter paper in a humidity chamber with continuous humidity of approximately 100% and at a temperature of 15–18°C. Mortality is recorded daily, every 24 h after the time of sampling, until 100% mortality is reached. This may take up to 25 d. Mussels are considered alive when closed individuals resist forcible valve separation. Dead mussels are always removed from the chamber, and the humidity chamber cleaned and refreshed daily with clean filter paper. The lethal threshold for 50% mortality (LT_{50}) and time to maximum mortality (TMM), both in days, is reported.

16.3 Confounding factors

Water and air temperature at the time of sampling should not be extreme (i.e. collection should not be carried out when environmental temperatures are close to zero or above 25°C) as this may influence the measurement. As a supporting parameter, condition index (CI) should be measured. Spawned out mussels with a low CI tend to be weak and will die quickly when measured for SoS. If information on spawning state is not known, SoS should not be carried out at or immediately after the main spawning season. Tolerance of small mussels to air exposure has been demonstrated to be significantly greater than for large mussels (R. E. Thomas *et al.*, 1999). To date, there is no evidence to suggest that there will be differences in SoS response for different species of mussels or hybrids.

16.4 Applicability across the OSPAR maritime area

SoS was also successfully used as a biomarker of stress in biomonitoring programmes such as RAMOGE (Mediterranean Sea). To date, SoS has not routinely been applied in the OSPAR maritime area, except for some research activities (e.g. Labarta *et al.*, 2005) and the ICON ICES/OSPAR Demonstration Programme (currently in progress). However, mussels are available throughout the OSPAR area, and there are no apparent significant constraints on their use. Several recent papers have emphasized the importance of using this simple biomarker to evaluate the effects of polycyclic aromatic hydrocarbons, for example around the "Exxon Valdez" site (R. E. Thomas *et al.*, 1999), for Halifax Harbour biomonitoring (Hellou and Law, 2003), and in studying the effects of the pollutants present in untreated sewage (Moles and Hale, 2003).

SoS has been adopted as a general stress biomarker in the UNEP MAP Mediterranean Biomonitoring Programme UNEP/MAP (UNEP, 2003). However, it could easily and equally be applied in the OSPAR maritime area as a whole-organism biomarker.

The measurement does not require sophisticated equipment and is low-cost in terms of manpower to undertake the work. Mussels can be collected from the shoreline or close to the shore, avoiding the high cost of research vessels. Therefore, the applicability across the OSPAR maritime area is highly recommended. Most importantly, it can be used as an index of a general stress syndrome within the integrated mussel monitoring framework proposed by OSPAR. Furthermore, SoS shows a sensitivity that is in the same range of other commonly used general stress indices at the cellular level (e.g. lysosomal membrane stability).

For the new organization of biological effects monitoring in Phase IV of the MED POL Programme, a two-tier approach has been proposed. This approach considers lysosomal membrane stability (LMS), stress on stress (SoS), and mortality as core biomarkers to be applied in the first tier.

16.5 Ecological relevance

This extremely simple biomarker is able to provide evidence of effects of pollutants at the whole-organism response level.

The response of this biomarker shows a typical dose–response curve, characterized by a continuous decrease in the parameter (LT_{50}) with increasing pollutant concentrations, although in some experiments in the presence of low concentrations of contaminants a slight increase was also observed, possibly the result of an hormetic effect (Eertman *et al.*, 1995).

16.6 Quality assurance

LT_{50} values have been reported to show comparability with stress indices determined at the cellular level (Hellou and Law, 2003).

Because of the simplicity of the method, data quality assurance has not been tested by national or international programmes and is not considered to be necessary (WGBEC, 2010, pers. comm.). However, an intercalibration/workshop exercise for other mussel techniques has been proposed in 2010, and it is proposed to include SoS in this initiative in order to harmonize the SOP and identify any Analytical Quality Control (AQC) issues.

16.7 Background responses and assessment criteria

Background response times and response thresholds corresponding to unintended/unacceptable levels of response have yet to be defined for SoS. Until more data are available, values should be interpreted from existing national datasets. It should be noted that these values are provisional and require further validation.

- Animals may be considered healthy if SoS is more than 10 d.
- Animals may be considered to be stressed but compensating if SoS is between 5 and 10 d.
- Animals may be considered as severely stressed if the SoS is less than 5 d.

Background SoS responses may be as high as 18 d, as observed in *M. galloprovincialis*, (C. Martínez-Gómez, pers. comm.) and 16 d in *Mytilus edulis* (J. Thain, pers. comm.).

The added value of SoS in mussels is that the response measures the overall impact of multiple stressors on an organism, yet the response can be correlated quantitatively to contaminant tissue concentrations, a "true" integrated biological effect–chemical monitoring tool (see ecological relevance above).

17 Background document: scope for growth in mussels (and other bivalve species)

John Widdows

17.1 Background

Growth provides one of the most sensitive measures of stress in an organism because growth integrates major physiological responses, specifically the balance between processes of energy acquisition (feeding and digestion) and energy expenditure (metabolism and excretion). Each physiological response can be readily determined in bivalves, converted into energy equivalents, and alterations in the energy available for growth and reproduction (scope for growth – SFG).

SFG, when used as an integrated approach to monitoring, is based on the combined measurement of SFG and chemical contaminants in mussels, and has been used successfully to detect, quantify, and identify the potential causes of pollution in estuaries and bays (typically over small spatial scales of ca. 10 km; reviewed by Widdows and Donkin, 1992), as well as over larger spatial scales of >1000 km of North Sea coastline (Widdows *et al*., 1995a) and the Irish Sea coastline (Widdows *et al*., 2002). Furthermore, SFG has been applied over a wide range of latitudes from subtropical (Bermuda; Widdows *et al*., 1990) to Subarctic (Iceland; Halldórsson *et al*., 2005).

Not only has SFG been correlated with concentrations of toxic contaminants in the tissues of mussels, but recent studies have also demonstrated that SFG correlates with measures of biodiversity in the benthic community (Crowe *et al*., 2004). Therefore, SFG can provide an effective indicator of pollution effects at both individual and community levels.

The methodology for determining the SFG of mussels (and other bivalve species) has been applied routinely to both toxicant-exposed mussels in laboratory studies and mussels collected from polluted environments. Laboratory studies are used to establish relationships between toxicant tissue concentrations and SFG, which can then be used to provide a quantitative toxicological interpretation of SFG and tissue contaminant levels in field monitoring programmes (Widdows and Donkin, 1992; Widdows *et al*., 1995a, 2002).

The underlying objective of both field and laboratory studies is to maintain and measure the SFG of individual mussels under "near optimal" conditions, so that the SFG will be maximized at a given ration level, and any reduction in SFG will reflect the stress induced by the toxicants accumulated in their body tissues.

The methodology for measuring SFG is well documented in the *ICES TIMES* series (no. 40). By standardizing the ration level in SFG measurements, the food-absorption efficiency remains relatively constant, and food is removed as a key variable, so allowing SFG to reflect the underlying impact of the total toxicant load accumulated within the body tissues (Widdows and Johnson, 1988; Widdows *et al*., 1990, 1995a,b, 1997, 2002). Although this SFG measurement does not predict the actual growth in the field, because food availability in the coastal environment is temporally and spatially variable and difficult to measure routinely, it does reflect the overall growth potential for individuals and mussel populations. For example, mussels from the Liverpool Bay and Morecombe Bay region of the Irish Sea had the lowest SFG values, and this was consistent with very low growth rates (an order of magnitude lower than unpolluted areas; Widdows *et al*., 2002). Subsequent studies by Crowe *et al*. (2004) have demonstrated a lower biodiversity within the mussel bed community at the Irish Sea study sites with low mussel SFG.

In addition, more detailed chemical analyses of the mussel tissues have confirmed that the lowered SFG and biodiversity values were correlated with increased concentrations of hydrocarbons accumulated in the mussel tissues, particularly those associated with the "unresolved complex mixture" (Crowe *et al*., 2004). Recent studies by Donkin *et al*. (2003) and A. Booth (pers. comm.) have begun to identify these previously unresolved compounds and shown them to be toxic to mussels.

This demonstrates that SFG is able to detect and quantify pollution impact and that subsequent independent studies were able to analyse and identify the nature of the toxicants in more detail and show changes at the population and community level.

17.2 Confounding factors

The preferred season for measuring SFG of field-collected mussels is during the period of maximum growth potential (i.e. from early summer to early autumn). It is important to avoid measurement of SFG or any other cellular/biochemical response during the spawning period, the timing of which is variable depending on latitude and seasonal temperature regime (but generally late winter / spring). The process of sampling and transportation of mussels at this time increases the probability of inducing the release of gametes (particularly following a prolonged period of air exposure or a temperature/physical shock), and this naturally stresses the animal. The release of gametes makes it very difficult to perform SFG measurements successfully. In addition, it is advisable not to measure SFG in autumn during a period of natural quiescence before the onset of gametogenesis.

Mussels chosen to represent clean reference sites in field studies should be collected from a location that is free from significant chemical contamination (i.e. removed from local sewage inputs, urban development, and industry). Mussels collected from the mouth of most estuaries are not representative of a clean reference site. It is advisable to analyse body tissues for contaminants, particularly organics such as hydrocarbons, to confirm that the site is not significantly contaminated. (Visual assessment of the site is not sufficient.)

Mussels used for SFG can be collected either from native populations or from specific sites where mussels from a clean reference site have been transplanted and exposed in cages for a period of >4 wk.

The basic physiological responses of mussels (such as feeding and respiration rate) remain relatively independent of short-term changes in natural environmental variables over a wide range of conditions; for example, food/seston concentration (0.1–20 mg l^{-1} seston; Widdows *et al*., 1979; Kiørboe *et al*., 1980), temperature (6–20°C; Widdows, 1976), and salinity (20–33; Widdows, 1985a). In addition, transplantation experiments over >1000 km have shown that any measurable differences in physiological responses and growth rates of different populations reflect environmental factors rather than genetic differences (Kautsky *et al*., 1990; Widdows *et al*., 1995a), permitting the direct comparison of mussels over a wide geographical area. This does not imply that genetically determined population differences in physiological responses do not exist, but that they are only apparent under extreme environmental conditions (e.g. elevated temperatures and reduced salinities).

17.3 Ecological relevance

Scope for growth (SFG) provides an instantaneous measure of the energy status of an animal, which can range from maximum positive values under optimal conditions, declining to negative values when an animal is severely stressed and utilizing body reserves. Although direct measurements of total production and growth rate are often difficult to quantify and interpret in relation to pollution (Widdows and Donkin, 1992), SFG is rapidly determined, providing a sensitive, quantitative, and integrated response that can be related to contaminant levels in body tissues. SFG has been applied in laboratory and mesocosm experiments to assess the toxic effects (from sublethal to lethal) of a range of environmentally important chemical contaminants, including aromatic and aliphatic hydrocarbons (Widdows *et al*., 1982; Donkin *et al*., 1989, 1991), sewage sludge (Butler *et al*., 1990), tri- and dibutyltin (Widdows and Page, 1993), nonylphenol (Granmo *et al*., 1989), pentachlorophenol (Widdows and Donkin, 1991), and organochlorine, organophosphate, and pyrethroid pesticides (Donkin *et al*., 1997). These laboratory studies have been particularly important in (i) establishing concentration–response relationships between the concentration of contaminants in the body tissues and the physiological responses of mussels, including SFG, and (ii) utilizing a quantitative structure–activity relationship (QSAR) approach to study the sublethal toxicity of organic contaminants (Donkin and Widdows, 1990). Such laboratory-derived, concentration–response relationships have been used subsequently to provide a quantitative toxicological interpretation of field-derived SFG measurements and tissue-residue chemistry (see Table 17.1).

There are many examples of the field application of SFG measurements combined with tissue-residue chemistry as a means of assessing environmental pollution. These include studies of pollution gradients in Maine (Gilfillan *et al*., 1977), Narragansett Bay (Widdows *et al*., 1981), San Francisco Bay (Martin and Severeid, 1984), the North Sea oil terminal in Sullom Voe, Shetlands (Widdows *et al*., 1987, 1995b), Venice Lagoon (Widdows *et al*., 1997), and two IOC GEEP workshops concerned with contaminant gradients in a Norwegian fjord (Widdows and Johnson, 1988) and Bermuda (Widdows *et al*., 1990). In addition, two field studies by Nelson (1990) and Anderlini (1992) have used SFG to assess the impact of sewage inputs to Narragansett Bay (USA) and Wellington Harbour (New Zealand), respectively.

More recently, the combined measurement of SFG and chemical contaminants in mussels has been successfully extended and applied over a larger spatial scale of >1000 km of North Sea (Widdows *et al*., 1995a) and Irish Sea coastline (Widdows *et al*., 2002). The main features of the approach in the North Sea and Irish Sea studies were to: (i) identify regions as well as specific sites that were significantly stressed by pollutants, (ii) quantify the degree of sublethal stress and how near the animals were to the lethal limit, and (iii) provide a quantitative toxicological interpretation of much of the contaminant data.

Therefore, these various field studies have demonstrated that this approach is able to detect and quantify changes in environmental quality, as well as identify some of the cause(s) of these changes through use of QSAR relationships and established cause–effect relationships (i.e. between contaminant concentrations in mussel tissues and the SFG response).

SFG has also been shown to be a sensitive and ecologically meaningful biological response that can provide a powerful, rapid (i.e. results can be obtained within days of sampling), and cost-effective method for monitoring changes in environmental quality (Widdows *et al*., 1995a, 2002; Crowe *et al*., 2004).

Table 17.1. Concentration–response and field-derived SFG measurements for various toxicants found in mussels

Toxicant	Tissue concentration inducing 50% reduction in feeding rate (CR) or SFG (mg g⁻¹ dry weight)	Reference
HMW Alkanes	>2 000 (CR)	Widdows and Donkin (1992)
HMW Aromatics	>2 000 (CR)	Widdows and Donkin (1992)
Lindane	1 400 (CR)	Donkin *et al.* (1997)
Cadmium	>150 (SFG)	Poulson *et al.* (1982)
Di(ethylhexyl)phthalate	330 (CR)	Donkin *et al.* (1996)
LMW Alkanes	125 (CR)	Donkin *et al.* (1989)
LMW Aromatics	125 (CR)	Donkin *et al.* (1989)
	20 (SFG)	Widdows *et al.* (1995a)
Carbaryl	50 (CR)	Donkin *et al.* (1997)
Pentachlorophenol	45 (SFG)	Widdows and Donkin (1991)
Copper	30 (SFG)	Widdows and Johnson (1988)
Tributyltin	4 (SFG)	Widdows and Page (1993)
Dichlorvos	2.2 (CR)	Donkin *et al.* (1996)

17.4 Quality assurance

The method has been successfully tested nationally in a range of UK monitoring programmes and internationally as part of IOC biological effects workshops to evaluate and compare pollution effects measurements at different levels of biological organization.

A successful intercalibration has been carried out across Europe to establish AQC procedures. This exercise provided the basis and mechanism for undertaking further intercalibration exercises. AQC is able to be provided through BEQUALM.

Health-status thresholds have yet to be defined for SFG, but from the extensive datasets that exist, the following values can be estimated, based on Widdows *et al.* (1995a,b, 2002):

- Animals may be considered to have low stress if the SFG is above +15 ($J\ h^{-1}\ g^{-1}$).
- Animals may be considered as moderately stressed if the SFG is between +5 to +15 ($J\ h^{-1}\ g^{-1}$).
- Animals may be considered to be highly stressed if the SFG is below +5 ($J\ h^{-1}\ g^{-1}$).
- Therefore the background assessment criterion (BAC) is set at +15 ($J\ h^{-1}\ g^{-1}$).
- The environmental assessment criterion (EAC) is set at +5 ($J\ h^{-1}\ g^{-1}$).

The added value of SFG in mussels is that the response measures the overall impact of multiple contaminants on an organism, yet the response can be correlated quantitatively to contaminant tissue concentrations, a "true" integrated biological effect–chemical monitoring tool (see ecological relevance above).

Furthermore, the recent work by Crowe *et al.* (2004) shows that SFG correlates with measures of biodiversity in the benthic community.

17.5 Acknowledgement

The majority of the above text was provided from the *ICES TIMES* (2006) guideline on the measurement of scope for growth in blue mussels provided by John Widdows and Fred Staff (Plymouth Marine Laboratory, UK).

18 Technical annex: integrated chemical and biological monitoring of mussel (*Mytilus* sp.)

John Thain, Concepción Martínez-Gómez, and Matt Gubbins

18.1 Background

The basis for this technical annex is the mussel integrated monitoring strategy incorporating biological effect techniques at the subcellular, tissue, and whole-organism responses and tissue chemistry. This is outlined in Figure 18.1.

18.2 Purpose of work

The integrated approach described above can be used for the following.

- Status and trend monitoring. Contaminant and biological effect responses are measured over geographic areas and repeated over time. The purpose here may be to (i) compare biological effect responses between sites, (ii) compare changes in response with time, and (iii) observe if the "health status" is improving at a steady state or declining.
- Investigative monitoring. This is most frequently used as a screening step to assess if biological effects are occurring in relation to a suspected contaminant gradient, pollution event, or if biological effects are suspected for any reason (e.g. tissue chemical residues have been observed to be high).
- Hot spot—site-specific monitoring. This is usually done in relation to risk assessment at pollution sites (e.g. oil platform investigations).

18.2.1 Offshore and coastal

Mussels (*Mytilus* sp.) are infrequently found in the sublittoral zone, but populations do exist in shallow water and are found on the seabed, usually close to the coastline, in general within the 12-mile limit. They may also be found offshore attached to navigation buoys, chains, and oil and gas platforms. For monitoring purposes, these mussels can be used, but care needs to be exercised in sampling the organisms to ensure that they are not damaged during sampling and that the correct size range can be obtained. For offshore monitoring purposes, it is usually more applicable to use *in situ* caging methods (see below). Advantages of using caged organisms are (i) choice of site deployment (including reference sites), selection of depth of deployment (e.g. may be critical for oil platform studies, but generally within 8 m of the sea surface); and (ii) standardization of origin (same source/supply), size, and species. Disadvantages are (i) cost of deployment in respect of mooring systems and ship time for deployment and retrieval; and (ii) some techniques require immediate sampling and analysis which may not be feasible on a research vessel offshore.

If caging is used, hydrographical conditions must be considered, with special attention given to water currents and stratification.

18.2.2 Shoreline

Mussels may be regarded as ubiquitous on rocky shore coastlines and, therefore, ideal for monitoring purposes. Sampling sites can be selected easily, organisms collected with little cost, and reference sites located without difficulty. In addition, if mussels are not present at a site of interest, organisms can be caged on the seashore or in estuaries on piers or similar structures.

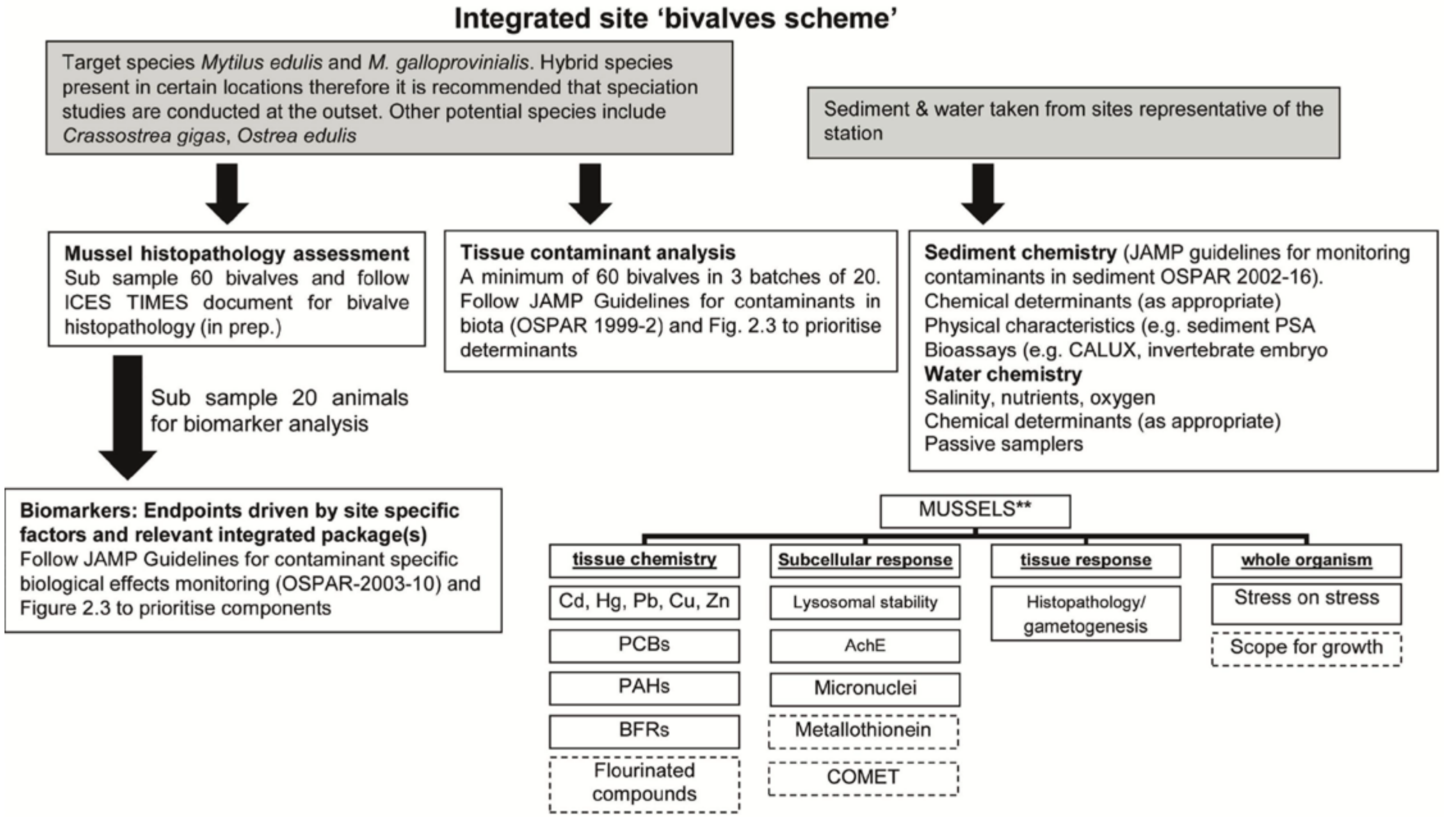


Figure 18.1. Sampling strategy for integrated bivalve monitoring.

** Figure 2.3 Overview of methods to be included in an integrated programme for selected bivalve species. (Solid lines – core methods, broken lines – additional methods).

In any mussel integrated monitoring programme, the core components indicated here should be included as a bare minimum.

18.3 Sampling information

The following details are required:

- Date, time, and location on the shoreline (if applicable, e.g. low water) and exposure (e.g. highly exposed Atlantic rocky shore or enclosed sheltered bay).
- Position in latitude and longitude.
- Type of site: reference, pollution gradient, status, or trend.
- At caging sites, information on water temperature, depth of deployment, time of immersion, water column depth, information on currents and stratification if available, and salinity.
- Source of mussels for caging studies: for any caging study, it is important that the mussels are sourced from a clean site, and that day-0 values are determined for tissue-contaminant chemistry and biological effect responses.
- For shoreline monitoring, the mussels must ideally be sampled in a uniform manner between sites (i.e. tidal height and similar salinity profile).

18.4 Confounding factors

- For in situ transplants/caging, the mussels must be deployed for at least 3 wk in order to allow sufficient time for contaminants to accumulate in the tissues and reach a state of equilibrium. Failure to do this may produce spurious data. Also of note is that, in many countries, there are regulations controlling the movement and deposit of shellfish, and these must be observed (i.e. prevention of transfer of disease).
- Reproductive state and gametogenic cycle: mussels generally spawn in early spring, with spawning occurring later in more northern populations. At spawning, there is a major loss in body lipid and a subsequent fall in condition; therefore, sampling in or shortly after this period should be avoided for all aspects of tissue chemistry analysis and biological effect determinations.
- Salinity: be aware that low salinities affect the biomarker response. This is of particular importance for caging work in estuaries.
- Temperature: mussels on the shoreline can be subject to extremes in temperature, cold in winter and extreme heat in summer. Avoid sampling when extremes are likely to occur, as this may compromise the biological effects response.
- Parasites: mussels with severe parasite infections should not be used.
- Algal blooms: in spring, late summer, and autumn, intensive algal blooms may occur, and sampling of mussels at such times should be avoided.
- Species: on some coastlines, mussels are solely of one species, whereas at other locations, they are mixed or hybrids. It is unclear whether or not species difference will affect interpretation of data, but wherever possible, attempts should be made to determine the species under observation.
- In caging studies (shoreline or offshore), care should be taken in sourcing mussels from a "clean site". If rope-grown mussels are chosen, particular attention must be given to transporting the mussels, as they tend to have weak adductor muscles and easily gape and become stressed during

transportation, which may give rise to initial mortalities or erroneous biological effect responses. Therefore, the source of mussels should be taken into account in the experimental design.

18.5 Supporting measurements

- Condition index: dry meat relative to whole live weight or internal shell volume.
- Gonad state: index of reproductive state.
- Lipid content: usually determined and measured along with tissue chemistry and useful for interpretation of biomarker responses.
- Real growth: if available, measured using growth of marked intervals over time, usually months.
- Water quality measurements: salinity, temperature are recommended, and where possible, suspended solids or turbidity, dissolved oxygen, and chlorophyll.
- Chemical analysis of tissues: this is essential for interpretation of biological effects data and for the implementation of the integrated chemical–biological effect strategy as outlined above. Prioritized contaminants are Cd, Cu, Hg, Zn, Cd, PAHs, and PCBs. As a minimum, 50 mussels (>40 mm in length) should be collected, taken to the laboratory, and held in running seawater for 24 h to eliminate gut contents (e.g. sediment). The tissues should then be extracted from the mussel and placed in acid-washed, hexane-rinsed glass/plastic/metal containers (as appropriate for the particular analysis), stored at –20°C for subsequent chemical analysis using ICES or appropriate protocols.

18.6 Sampling for biological effects

For some methods, the samples require immediate processing at the time of sampling, whereas for other techniques, processing is undertaken in the laboratory. An overview of this is shown in Table 18.1, and also includes the number of animals typically sampled for each method. Ideally, the size of individual mussels for all methods is >40 mm.

Table 18.1. Overview of sampling procedures for mussels

Method and minimum numbers of animals usually sampled per site in brackets	When analytical sampling is undertaken	Acclimation	Comments and aspects that are crucial
SFG (10)	24 h	ca. 10 h	Crucial
AChE (10)	Immediate in field	Not applicable	Stored immediately in liquid nitrogen
Metallothionein (10)	Any time within 24 h on live mussel	Not applicable	Take tissue sample—freeze in liquid nitrogen
Comet	Within 24 h	Store for no more than 24 h in cool damp conditions. Must be consistent in strategy	Do as quickly as possible
Micronuclei (20)	Within 3 d	None	Mussels can be kept out of water but cool
NRR (10)	Within 24 h	Store for no more than 24 h in cool damp conditions. Must be consistent in strategy	Do as quickly as possible

Lysosomal histochemical method (10)	Freeze immediately	Not applicable	In liquid nitrogen
Stress on stress (40)	Not applicable	Transport at low temperatures for no more than 24 h	Analysis done at 18°C
Histopathology and gametogenesis (30–50)	Sample immediately if possible	Anything more than 6 h delay in sampling place in water for 48 h acclimation	Dessication must be avoided, correct dissection to incude all organs
Tissue chemistry (50)	Place in clean running seawater for 24 h	Not applicable	Depuration of sediment is crucial

Mussels are attached to each other or to a substrate by a byssal thread. When mussels are sampled, care should be used not to pull the mussels and byssal threads too vigorously as this can damage and stress the mussels. If mussels have to be transported, this should be kept to a minimum, and they should be kept damp and cool and, if possible, the temperature logged during the transport.

For some techniques, such as SFG, the mussels will need to be carefully cleaned. It should be noted that there are limitations of analysis for some methods (e.g. for SFG and NRR), where, time-wise, it may be difficult to process more than two samples in a single day.

For histological sampling, it is essential that the dissection is conducted in a precise manner as described below.

The technical procedure essential to correct mussel sampling for histology (taken from a draft manuscript intended for future *TIMES* publication, provided by J. Bignell UK, Cefas):

1. Insert scalpel into ventral byssal cavity and move knife down so it cuts the posterior adductor muscle.
2. Open shell and remove byssal thread.
3. Remove mussel from one shell half. Repeat for remaining half.
4. Analyse tissue for presence of parasites, pearls, or other abnormalities.
5. Obtain a standardized section, as shown in Figure 18.2, in order to include all organs of interest in one section, and place into histo-cassette.

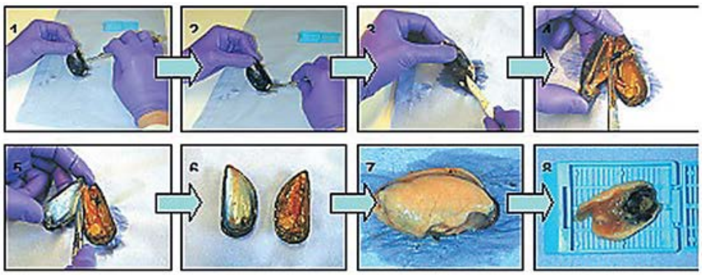


Figure 18.2. Obtaining a standardized section from mussel.

6. Samples should be preserved for a minimum of 24 h in Baker's Formal Calcium, and subsequently transferred to 70% alcohol until processed.
7. The correct ratio of mussels to fixative is 30 samples per 800 ml (approximately) of fixative. This is the recommended volume of fixative to ensure adequate fixation.
8. Samples should be agitated periodically to ensure thorough fixation. A rocker plate facilitates this perfectly.

18.7 Methods to be used

These are listed in the mussel integrated strategy above. An overview of the methods is given in Table 18.2, with references to the analytical procedures.

Table 18.2. Overview of methods and reference to analytical procedure

Method	Issue addressed	Biological significance	References
AChE inhibition	Organophosphates and carbamates or similar molecules. Possibly algal toxins	Measures exposure to a wide range of compounds and a marker of stress	1–2
Metallothionein induction	Measures induction of metallothionein protein by certain metals (e.g. Zn, Cu, Cd, Hg)	Measures exposure and disturbance of copper and zinc metabolism	3–4
Lysosomal stability (including NRR)	Not contaminant-specific, but responds to a wide variety of xenobiotic contaminants and metals	Measures cellular damage and is a good predictor of pathology. Provides a link between exposure and pathological endpoints. Possibly a tool for immunosuppression studies in white blood cells	5–19
Scope for growth	Responds to a wide variety of contaminants	Integrative response, a sensitive sublethal measure of energy available for growth	20–21
Stress on stress	Responds to a wide variety of contaminants and other environmental conditions	Integrative response, a measure of stress, condition, health, and well-being	26
Micronuclei	Exposure to aneugenic and clastogenic	Exposure to aneugenic and clastogenic	22–23
Histopathology and gametogenesis	Not contaminant-specific	General responses	24–25++
Comet	Genotoxic compounds	DNA strand breaks	See OSPAR background document

18.8 Quality assurance

Wherever possible, all analytical methods must be supported with quality assurance (QA) procedures. These should be through international intercalibration exercises where they exist and through internal quality controls.

The current position with quality assurance is:

- NRR—currently being developed across OSPAR, exists in MED POL, for internal QA a dual assessment with a colleague on the same samples is recommended.
- AChE—not yet developed, but include internal standard.
- MT—MED POL has intercalibration exercises, elsewhere there have been *ad hoc* intercalibrations, and additionally an internal standard should be included.
- SFG—none at present.
- Stress on stress— addressed by ICES/OSPAR Workshop on Lysosomal Stability data quality and interpretation and associated MED POL Training Workshop in 2010

- Histology and gametogenesis—*TIMES* document and circulation of reference material.
- Lysosomal histochemical procedures—none currently available, but include an internal standard. Addressed by ICES/OSPAR Workshop on Lysosomal Stability data quality and interpretation (WKLYS) held in 2010
- Micronuclei formation—currently being addressed through MED POL and may be extended to include a wider participation.
- Comet—none at present, but being addressed through ICES WGBEC.

18.9 Reporting requirements

- **Biological effect responses.** These should be reported in line with requirements detailed in each analytical method. When different biological effect measurements are made on the same individual mussel, the data should be identified in the reporting and data assessment.
- **Contaminants.** These should be reported in line with standard analytical procedures.

18.9.1 Supporting parameters

- Essential: Date and time of sampling, latitude-longitude position, organism length, whole weight, site characterization (e.g. position on shore, or caging, dissolved oxygen, salinity); for caged studies, the source of organisms and duration of exposure.
- Desirable: identification of species, particularly if in a hybrid zone.

19 Background document: water *in vivo* bioassays

Dick Vethaak, Ricardo Beiras, and John Thain

19.1 Executive summary

Applicability across the OSPAR maritime area. Water *in vivo* bioassays are available for immediate deployment within the OSPAR Joint Assessment and Monitoring Programme (JAMP) Coordinated Environmental Monitoring Programme (CEMP). These bioassays have been recommended by ICES and are of sufficient standing in terms of methodological development, ease of use, and application for uptake across the whole OSPAR area. The preferred method is short-term tests on concentrates of water. This includes broad-spectrum (acute and short-term chronic) bioassays (and can be combined with specific *in vitro* bioassays), which can be applied to salt water, brackish water, and freshwater, allowing all types of water to be assessed in the same way, thereby giving a comprehensive picture of an entire area. If the focus is also on specific groups of substances or a specific toxicity, such as hormone-disrupting effects or neurotoxicity, *in vitro* bioassays can be used on concentrates or otherwise. Chronic (long-term) *in vivo* bioassays would appear to be most suited to site-specific assessment and comparison with the field situation (e.g. to provide sufficient evidence to support the conclusion that a problem no longer occurs). The long-term exposure without concentration of the sample means these tests give the most realistic estimate of the possible effects in the field. Relevant acute bioassays can be a quick and cheap alternative, as can *in vitro* tests.

Water bioassays should be deployed as a “battery of tests” and should include a minimum basic set, possibly of three or more. However, the composition of what the set needs to comprise requires further work. The range of bioassays needs to be expanded to include all trophic levels and phyla, such as echinoderms.

Quality assurance. QA procedures are in place for most of the (water) bioassays and are provided for by BEQUALM (www.bequalm.org); therefore, bioassay data can be submitted to the ICES database for subsequent assessment, as appropriate, by ICES/OSPAR. A standardized protocol for bioassay extractions is required to ensure consistency of application between laboratories and Member States and comparability of reported data for assessment purposes and will be published as an *ICES TIMES* series document. **Influence of environmental variables**. Abiotic testing conditions, such as temperature, salinity, solids, dissolved oxygen, and pH, can dramatically influence test variability. The same is true for the condition and age of test organisms and storage conditions of test samples. In general, these factors are standardized in the test procedures and controlled during the test period by the use of positive and negative controls. The use of extracts/concentrates will further reduce any disturbing factors.

Thresholds and assessment tools. Three assessment classes were derived for water bioassays: (i) a background response, (ii) a warning level, and (iii) a level of serious concern. The background responses for the water bioassays (*Tisbe* sp., *Acartia* sp., sea urchin, and bivalve larvae) were 10, 10, 10, and 20% mortality (or deformity as appropriate), respectively. The level of serious concern was between twofold the background response and 100% mortality, and the warning level between these values.

In this report, we describe and propose an ecotoxicological metric for acute and chronic *in vivo* bioassays. An acute/chronic ratio of 10 is used to convert the acute

data to chronic data. If data are available from three bioassays, a preliminary effect assessment can be performed. If at least four chronic values are available for different taxonomic groups, a refined effect assessment can be carried out whereby the potentially affected fraction (PAF) approach is used to calculate the percentage of affected species in the ecosystem in question. With its ”negligible effect”, ”maximum permissible effect”, and ”serious effect” classification, this method assessment is consistent with the current Dutch standard framework and terminology (environmental risk limits). It is, however, equally suited to the current OSPAR and EU WFD (Water Framework Directive) assessment frameworks.

Synergism between CEMP and WFD. There are clear opportunities for synergism between the CEMP and WFD for water bioassay applications in coastal and estuarine areas, but further work and agreement are needed.

Recommendations. The sampling strategy and design of water quality monitoring for spatial and temporal monitoring purposes needs to be clearly defined and, in particular, the role of water concentrates. In this respect, there is an important need to develop and validate appropriate protocols for extraction methods and subsequent *in vivo* (and *in vitro*) testing. More research is also needed to link bioassay responses to actual impacts on the aquatic system. The application of passive samplers for bioassay assessment of water also warrants special attention.

19.2 Assessment of the applicability of water *in vivo* (and *in vitro*) bioassays across the OSPAR maritime area

Most existing bioassays have been used for reporting to regulatory commissions on individual hazardous substances and the determination of environmental quality standards (den Besten and Munawar, 2005). Over the past few decades, bioassays have also been used for the risk assessment and management of saline and freshwater whole effluents (e.g. Oris and Klaine, 2000; Power, 2004), and for dredged material (e.g. Stronkhorst *et al*., 2003).

To date, there are numerous studies illustrating the application of bioassays to assess the toxicity of environmental samples from marine and inland surface water (e.g. Karbe, 1992; Hill *et al*., 1993; Matthiessen *et al*., 1993; Hendriks *et al*., 1994; Kirby *et al*., 1998; K. V. Thomas *et al*., 1999; Peters *et al*., 2002; Åkerman and Smit, 2003; Derksen *et al*., 2004). For example, bioassay assessment of fresh surface water has been used successfully for many years in the Netherlands in the context of the surveillance monitoring of the Meuse, Scheldt, and Rhine river basins (Maas *et al*., 2003). This assessment used acute bioassays (or *in vitro* bioassays; including CALUX systems, Microtox, *Daphnia* and whole sediment, pore water) on XAD concentrates of the water (e.g. Hendriks *et al*., 1994 Maas *et al*. 2003). The ICES/IOC Bremerhaven Workshop on Biological Effects of Contaminants in the North Sea and the ICES BECPELAG Workshop on Biological Effects in Pelagic Ecosystems have clearly demonstrated the potential applicability of a variety of *in vivo* bioassays to coastal and offshore water column and microsurface layer monitoring (Stebbing *et al*., 1992; Hylland *et al*., 2002, 2006b).

Water bioassays recommended for use in different monitoring strategies are well described in OECD, ASTM, ISO, SETAC, and ICES test protocols (see also US EPA, 1995; Tonkes *et al*., 2005). Bioassays are widely recognized within Europe to be an efficient way to assess water quality. Bioassays are also applied at the national level by several countries (ICES, 2004). The uptake of water bioassays, such as the oyster embryo assay (Thain, 1991), in monitoring programmes across the OSPAR maritime area is, however, still poor (so far, only UK; see ICES, 2004). *In vivo* bioassays (and *in vitro* tests with microorganisms) are now also frequently used as tools in estimating the potential risk of contaminants of estuarine and marine waters (e.g. Murk *et al.*, 2002; Thomas *et al.*, 2002; Åkerman *et al.*, 2004).

The standard for bioassays described and proposed is based on a report produced by the Dutch Ministry of Transport, Public Works and Water Management/RWS (Maas *et al.*, 2003) and is primarily intended as a step towards the incorporation of biological effect assessment (bioassays in this case) into the CEMP, as desired within OSPAR.

The following definitions and terminology are used.

- Bioassays can be divided into *in vivo* and *in vitro* bioassays. A distinction can also be drawn between broad-spectrum bioassays and bioassays based on a specific action mechanism.
- In *in vivo* bioassays, whole living organisms (including bacteria) are exposed to environmental samples or extracts of samples. The tests may be of short duration (lasting several hours to several days) and designed to identify acute effects, or of longer duration (days or months) to determine chronic effects. They can be carried out in a laboratory or in the field (*in situ*). The effects noted, known as "endpoints", are compared with the endpoints of a control test. *In vivo* bioassays have been developed so as to provide broad-spectrum analysis.
- *In vitro* bioassays, such as DR-Luc/DR-CALUX, are laboratory tests using prepared cells or subcellular fractions isolated from organisms or modified bacteria. These tests are mechanism-based, of short duration (lasting from several minutes to several days), quick to perform, and small scale.
- Acute tests provide an initial screening, are of short duration, and identify "crude" effects, such as the death of the test organism. They simulate a "realistic worst-case" scenario: a one-off, short-term exposure to relatively high concentrations of pollutants.
- Chronic tests are designed to emulate the actual situation more closely: longer exposure (i.e. for a substantial proportion of the lifetime of the test organism) to lower concentrations. Endpoints include reduced reproduction or growth in the test organism. Chronic tests are generally more sensitive, but they are also more expensive and more complex in practice than acute tests.

The decision whether or not to perform an acute or chronic test will depend on the degree of pollution in the compartment. In surface waters, for instance, acute effects can be observed near point sources and after incidental adverse events; however, in salt water and freshwater, it is usually only possible to observe chronic effects. In cases where neither chronic nor acute effects have been measured, but there is a need to identify trends in toxicity or show the current level of toxicity, acute tests can be performed on concentrates of surface water. However, it must be remembered that not all substances can be concentrated to the same degree using the techniques available.

The advantages of acute tests are that several tests can be performed simultaneously, they produce rapid results, a smaller sample volume is needed, and they are generally cheaper. Water samples are also more constant in acute tests than in chronic tests.

In vivo and *in vitro* bioassays each have their own specific strengths and weaknesses. *In vivo* assays use the entire organism. The exposure situation in such tests is more consistent with the actual situation than in tests where only parts of organisms are used. Processes that play a role in toxicity, such as biological availability, metabolism, and bioaccumulation, can, therefore, be included.

The advantage of chronic *in vivo* bioassays is that they indicate potential longer-term effects. However, some chronic tests take a great deal of time, space, manpower, and money. This applies particularly for larger, longer lived organisms such as fish. However, some chronic tests can be completed within a fairly short time and cost little more than acute tests. They include growth inhibition tests on bacteria.

19.3 Preconditions and criteria for *in vivo* and *in vitro* bioassays

To ensure their application and acceptance, it is important that bioassays conform to certain criteria and include factors such as *relevance* and *reliability*, for example.

The requirements for recommending a bioassay for JAMP purposes have been proposed by ICES and must include inter- and intralaboratory quality assurance procedures. These are provided using agreed international procedures and through BEQUALM and intercalibration exercises. Several further requirements are listed and discussed below. The basic principle is that these tools should allow the ecosystem to be protected as much as possible. The ideal set of bioassays would be representative of all organisms and trophic levels in the ecosystem in question and that the most sensitive species are used. The ecosystem as a whole will be protected if a number of "trigger species" from several taxonomic groups are protected. Furthermore, in such an ideal situation, the response from the set of bioassays should allow all possible substances to be covered at both the acute and the chronic levels. The set should, therefore, also have the following qualities.

19.3.1 Ecologically and/or toxicologically relevant

Relevance refers to the guarantee that the bioassay will measure the toxic and ecological effect of interest. Relevance is determined, among other things, by the test's sensitivity, specificity, and discriminatory capacity. Ideally, the measured effect should be ecologically relevant; if the species is of ecological/commercial importance, this would be an additional advantage. Bioassays are "merely" a model of reality. The ecological relevance, in particular, of *in vitro* assays is the subject of debate. We also know too little about how to link the effects at bioassay level with real impacts on the aquatic system. Results from a combined set of bioassays (both *in vivo* and *in vitro*) might, however, provide evidence as to the ecological relevance of the observed effects.

19.3.2 Representative of all organisms and trophic levels in the ecosystem in question

There is currently no bioassay that is representative of all organisms and trophic levels. This means that a set of bioassays is always needed to cover the ecosystem as fully as possible. Ideally, this set would consist of bioassays for every class of organism: algae, bacteria, crustacea, mollusca, pisces, aves, etc. In line with the guidelines used in chemical standard-setting, at least three or four different taxonomic groups, at least one of which must be vertebrate, and a set of at least three or four *in vivo* bioassays would be needed, one of which used fish.

19.3.3 Covering all effects of all possible substances and action mechanisms, both acute and chronic

In vivo bioassays are whole-organism tests and, therefore, by definition, respond in an integrated manner to all of the contaminants that are present in a test sample (i.e. tests lack specificity, but have high relevance). At the moment, there is no *in vivo* bioassay that could be used to detect all possible mechanisms of toxicity, and no *in vitro* bioassay that is capable of detecting all substances or possible action mechanisms. The best way to address this issue is to use a set of *in vivo* and *in vitro* bioassays that cover as many different action mechanisms as possible (see de Zwart and Sterkenburg, 2002). However, some action mechanisms are not covered fully by *in vivo* bioassays, either because the tests are less sensitive, or because the effect occurs only after long-term exposure. This applies particularly to genotoxicity, immunotoxicity, hormone-disrupting effects, and dioxin-like toxicity, as well as the initial signs of neurotoxicity. Effects via these mechanisms are more likely to be detected with *in vitro* bioassays.

19.3.4 Sufficiently sensitive, specific, and discriminatory to predict effects

Some bioassays are very sensitive to very small quantities of contaminants in the tested material. This is particularly true of *in vitro* tests, which can respond specifically to a particular contaminant or have specific modes of action. Sometimes, an effect found in an *in vitro* test cannot be replicated in an *in vivo* bioassay. In such cases, the *in vitro* assay is probably too unspecific, so that it also responds to non-active substances present either naturally or otherwise in the matrix. The reverse also occurs: no response *in vitro*, response *in vivo*. In this case, it might be that the *in vitro* bioassay is too insensitive, or that there has been a loss of compounds during the exposure or processing of the environmental sample. In conclusion, all scenarios can be obviated by using a battery of test methods or a targeted bioassay when prior knowledge of the presence of a contaminant is suspected. The bioassay methods described above are well tried and intercalibrated and, as such, the inherent variability of the endpoints of each assay is well documented. Therefore, it is possible to design sampling and test strategies with adequate replication to provide good discriminatory power between test samples.

19.3.5 Reliable and reproducible

The reliability or precision of a bioassay relies on its reproducibility within the same laboratory, or in other laboratories (intra- and interlaboratory reproducibility). Reproducibility is determined by the stability of the bioassay. A standardized method laid down in a protocol with validity criteria and control for modifying factors is essential to a stable bioassay. All bioassay tests now use positive controls; this consists of a standardized reference material, which is run alongside the test samples and ensures that the response of the assay organism and the conditions are valid for the test.

19.3.6 Availability of test species

For the widespread use and acceptance of a bioassay, it is essential that the test organism is widely available geographically and that the species either can be collected easily and cheaply from the wild or is easily cultured in the laboratory. Care also needs to be taken to ensure that too much inbreeding in cultured organisms or seasonality in wild-collected organisms does not affect the response of the assay, but this should be taken account of if positive controls are employed.

Clearly, when compiling a set of bioassays for assessing the quality of water, one must also take into account other financial and practical considerations. Further conditions, therefore, include the following factors.

19.3.7 Financial considerations

In general, bioassays are not expensive (relative to other methodologies), and their incorporation into the CEMP should not entail excessive cost. Although it is not possible to specify any particular sum, it is realized that expensive bioassay packages that could include long-term exposure with chronic endpoints will have little chance of successful introduction and should be confined to targeted and site-specific problems.

19.3.8 Laboratory availability

The introduction of bioassays into the CEMP will place major demands on the available laboratory capacity. This capacity should, therefore, ideally be expanded. There should preferably be more contract laboratories that can routinely perform bioassays. The bioassays recommended in the JAMP CEMP have well-documented protocols, and the procedures are easy to learn and, in most cases, do not require expensive or sophisticated equipment or capital expenditure. Current methods tend to be microscale in operation, which, by definition, require less space and are more cost-effective.

19.3.9 Use of test animals

Societies across Europe wish to reduce the use of test animals, particularly vertebrates like fish. This trend is only likely to strengthen in future, meaning that *in vivo* bioassays with invertebrate organisms are preferable, and that more effort must be focused on the development of *in vitro* bioassays.

19.3.10 Availability of tests and incorporation into metric

By no means all of the promising tests have been worked out to the extent that they can be included in a set of biological effect instruments. The results of the CEMP bioassays in the set must, of course, be consistent with the proposed metrics.

Taking account of these extra conditions will allow a pragmatic set of bioassays to be selected from the ideal, scientifically sound set of bioassays. Ideally, this set should include a minimum of three acute or chronic *in vivo* bioassays on at least three different taxonomic groups, preferably not using vertebrates, and one or more *in vitro* bioassays.

19.3.11 Towards a normative framework for bioassays

The proposed framework for bioassays should preferably be generic, tying in readily with existing policy frameworks and with national and international criteria. An entirely new and unknown system would not be desirable. On the other hand, however, it must be possible to estimate location-specific risks.

It is usually necessary, when conducting rapid, acute *in vivo* tests and *in vitro* tests on surface waters, to produce a concentrate of the surface water. This is necessary because the concentration of contaminants in the bulk water is not acutely toxic; exceptions may be samples taken in estuaries or close to discharge points. Typically, a seawater concentrate is a method whereby contaminants are selectively extracted from a surface-water sample (e.g. 100 litres) onto a medium; the medium is eluted with an appropriate solvent, evaporated to a small volume, which is subsequently taken back up in seawater (e.g. 100 ml). In this example, a 1000-fold concentration of extractable contaminants and dilutions of this concentrate are bioassayed.

19.3.12 Advantages and disadvantages of working with concentrates

All kinds of confounding or interfering factors are automatically removed from the test sample during the extraction procedure. They include a high ammonium content, salinity, a high or low pH value, any ion imbalance, and hardness. The great advantage is that all water types, freshwater, salt water or brackish water, can be tested using the same (freshwater or salt water) methods. This allows one to obtain a picture of the entire OSPAR area, for example, and to compare all locations. Concentrates can be diluted again, so it is almost always possible to obtain a quantitative measure of the toxicity. Using a selective extraction method allows one to determine the cumulative effect of an entire group of substances with the same action mechanism, such as substances with an oestrogenic effect.

Bioassays conducted on surface water samples generally use a small sample volume, typically 20–100 ml taken from a discrete water sample of about 2 litres. Water extraction procedures require a larger sample volume (e.g. 100 litres), which can be regarded as a more representative and integrated sample. Furthermore, a greater integration can be achieved by taking samples over time, and subsequently bulking the water samples prior to extraction.

A major advantage of water extraction techniques is that a positive bioassay response can be followed by bioassay-led TIE (toxicity identification evaluation; US EPA, 1991, 1992, 1993) procedures. This is a procedure whereby a targeted bioassay response and targeted analytical chemistry can be used to identify the type or, in some cases, the specific compound causing the reduced water quality.

There are also drawbacks, however. Usually only a proportion of the substances is extracted, and the efficiency of the extraction process will depend on the medium and solvent used. Metals, in particular, tend to be ignored in the current procedures. This restricts our view of the total toxicity of the surface water, forcing us to overlook the combined effects of several substance groups with different action mechanisms, such as metals and organic micropollutants. The current extraction methods would appear to be broad enough for organic micropollutants. If not, two extracts can be mixed together, broadening the range of extracted substances. Passive samplers should be considered for the assessment of contaminant concentrations in water (replacing water samples); extracts from passive samplers could then be used for acute *in vivo* bioassays and *in vitro* bioassays. This approach could be used to detect the presence of new chemicals in areas selected for such monitoring. For more discussion of extraction methods, see ICES (2005a).

Chronic *in vivo* bioassays would seem to be most suited to site-specific assessment and comparison with the field situation. Long-term exposure without concentration gives the most ecologically realistic estimate of possible effects in the field. Appropriate acute bioassays, such as fertilization and embryo development tests, can be a quick, cheap alternative, as can *in vitro* tests.

19.4 Introduction of water *in vivo* bioassays to the CEMP and status of quality assurance

ICES agreed on the following revised criteria for recommended monitoring methods:

- A recommended method needs to be an established technique that is available as a published method in the *TIMES* series or elsewhere. This applies to both the bioassay itself and the preparation phase (such as the sampling and extraction methods).
- A recommended method (or combination of methods) must have been shown to respond to contaminant exposure in the field.
- A recommended method (or combination of methods) must be able to differentiate the effects of contaminants from natural background variability.

The OSPAR JAMP CEMP lists water bioassays as Category II rated. The corresponding technical annexes to the JAMP Guidelines for General Biological Effects Monitoring relate to the following bioassay methods: *Tisbe battagliai*, oyster embryo, *Nitocra*, and *Dinophilus*. However, other species are now also appropriate and have been recommended by ICES, including the methods turbot juvenile acute, *Daphnia* acute and chronic, *Acartia* acute, and *Skeletonema* 72-h growth.

Quality assurance through BEQUALM is in place or currently running (JAMP, 1998; ICES, 2005a). So far, uptake of water bioassays in BEQUALM has been slow, but is increasing. Protocols exist for water extracts, but they have not been agreed, standardized, and "transcribed" into OSPAR guidelines. A standardized protocol for bioassay extractions is required to ensure consistency of application between laboratories and Member States and comparability of reported data for assessment purposes. Also, these protocols are used as standard procedures for BEQUALM intercalibrations. The protocol for extraction methods for bioassays will be published in the *ICES Techniques in Marine Environmental Sciences* series on biological effects of contaminants.

19.5 Synergism between CEMP, Marine Strategy Framework Directive (MSFD), and WFD

Although bioassays are not included as ecological quality elements in the monitoring for the WFD (CIS, 2003), it is generally accepted that they will be able to contribute to investigative monitoring and to the pressures and impacts/risk assessment process (this is especially true of chronic water and sediment bioassays). This process, being carried out by national authorities, is designed to identify water bodies at risk of failing to achieve good ecological status. Further chemical analysis can be combined with water bioassays at smaller interval time-points for the purposes of trend monitoring. In this way, bioassays can be used as a partial replacement for chemical analysis of priority and/or other relevant substances and prioritizing locations for further chemical analysis. This "bioanalysis approach" can lead to more cost-efficient and cost-effective monitoring and would put the precautionary principle called for in the WFD into practice. Pilot studies carried out in the Netherlands to explore these possibilities have had promising results (van de Heuvel *et al*., 2005; Maas and van den Heuvel-Greve, 2005). It can be concluded that clear opportunities exist for synergism between the CEMP or the MSFD and WFD for bioassay applications in coastal and estuarine areas, but that further work and agreement are needed.

19.6 Thresholds and assessment tools

Thresholds for water bioassays are available. Effects measured include acute (e.g. mortality) or chronic endpoints (sublethal endpoint such as growth, development, and reproduction) and hence are generic indicators of toxicity of the water. Values of EC_{xx}, LC_{xx}, NOEC (no observed effect concentration), and LOEC (lowest observed effect concentration) are usually used where appropriate to evaluate the test responses and to estimate toxicity. Results of bioassays from a contaminated area can be compared with a reference area, in a dose–response relationship between sites or by using time-series analysis, multivariate analysis such as principal component analysis (PCA), and toxicological risk-ranking methods (e.g. Hartwell, 1998; Péry *et al.*, 2002). Ecotoxicological assessment criteria for water *in vivo* bioassays will also need to be developed for data derived from bioassay-directed water-extract testing.

Water *in vivo* bioassays include techniques that use specific testing regimes and species. Therefore, for the purposes of developing background responses and assessment values, each technique will require separate review.

19.7 Methods for water *in vivo* bioassays currently in JAMP

The species recommended for water *in vivo* bioassays:

- Copepods (*Tisbe battaglii* and *Acartia* sp): 48-h exposure using mortality as the endpoint.
- Bivalves (*Crassostrea gigas*, *Mytilus* spp.) embryos: 24-h exposure using per cent net response as the endpoint.
- Sea urchin (*Paracentrotus lividus*): 24-h embryo exposure using per cent normal development and larval length as the endpoints.

The methodology for water bioassays is well developed and available through *ICES TIMES* and/or OECD. Quality assurance is provided via BEQUALM for the bivalve tests and *Tisbe* assay.

In all water bioassays, control and positive control are used. The control is a "pristine water" of known water quality and characteristics (i.e. no contamination, full salinity, appropriate pH and dissolved oxygen, e.g. natural seawater from the Atlantic from ICES reference station or Cape Wrath). The control water is used in all tests, and test animal responses in all field and test samples are compared with the test animal response in the control water. A positive control is always used in each experimental design to assess the performance of the testing procedures, including the sensitivity of the test organism. The positive control consists of the control water spiked with a reference substance (usually a Zn salt). A reference water may also be included for site-specific programmes and may be considered as the control water for the sampling area or region under investigation and ideally should give the same response as the control water.

The methodology for the extraction or concentration generally requires sample manipulation and/or concentration techniques, and clean-up using extraction procedures analogous to those used in chemical analysis. These procedures and QA are being developed and documents will be published in the *ICES TIMES* series.

19.8 Assessing the data

The data for water bioassays can be considered in much the same way as for sediment bioassays. The background response is defined as the upper level of natural variation and can be determined as a percentile (for instance, 90%) of the individual responses (mortality or malformation) of the control water.

From experience in the UK, the Netherlands, and Spain, the maximum background level response is of the order of 10% for *Tisbe* sp. and *Acartia* sp. bioassays, 10% for sea urchin, and 15% for the bivalve embryo bioassay. These figures, however, need to be defined and further established when further data become available (see also

Table 19.1 below). Responses greater than twice these values and up to 100% are categorized as a level of serious concern (i.e. malformation and mortality is regarded as a serious, high-level individual population response). Data in this response range should trigger immediate follow-up investigations. Responses between background and twice the background should be categorized as a cause for concern and prompt further sampling in terms of geographical spread and frequency of sampling (possibly time-integrated water sampling). Responses at the serious concern level would initiate further assay of the water-test samples using a dilution series in order to quantify the toxicity using an EC_X (per cent dilution causing an x% reduction in the endpoint) or toxic units ($TU = 100/EC_X$) approach. A phased toxicity identification evaluation (TIE) can be conducted to further describe the nature of the toxicity or potential toxicants present.

19.9 Assessment of background response level of available data for water bioassays

A derivation of background response levels was attempted for the water bioassays using *Tisbe battaglii*, bivalve embryo, and echinoderm embryo. Data from controls were collected for several tests from different sources. When individual datasets were obtained, these were averaged per sample and listed in a database with standard deviation (see Table 19.2). From resulting samples, the average laboratory/country was calculated together with the 0.1, 0.5 (median), and 0.9 quantile. Where more datasets were available, the same was done with laboratory/country datasets. The current assessment thresholds are given in Table 19.1.

Table 19.1. Assessment criteria for water *in vivo* bioassays

BIOLOGICAL EFFECT	QUALIFYING COMMENTS	BACKGROUND RESPONSE RANGE	ELEVATED RESPONSE RANGE	HIGH AND CAUSE-FOR-CONCERN RESPONSE
Bioassays; % mortality	Water, copepod	0–10	>10–<50	>50
Bioassays; % abnormality	Water, bivalve embryo	0–20	>20–<50	>50
	Water, sea urchin embryo	0–10	>10–<50	>50
Bioassay; % growth	Water, sea urchin embryo	0–30	>30–<50	>50

Table 19.2. Template of data used for calculations of background responses for water bioassays (median, min and max are optional)

TEST	NAME OF THE TEST
Reference	Reference to the origin of the data
Year	Year of production
Country	
Laboratory	Laboratory that performed the analyses
Type	Is it a control or other type of sample
Endpoint	Type of measurement
Unit	
idnr	Sample number within a dataset
Replicates	Number of replicates
Result	Average value of the control
Median	Median of the individual data
Min	Minimum of the individual data

Max	Maximum of the individual data
s.d.	Standard deviation of the individual exposures

19.10 Ecotoxicological assessment criteria for *in vivo* and *in vitro* bioassays

This method is available, but needs further validation before it can be implemented.

19.11 Assessment framework: metric and criteria

The premise of the effects-oriented track for water and sediments is that exposure to substances should not result in "adverse" effects on humans and ecosystems. The metric should, therefore, be consistent with the environmental risk limits (ERLs) for individual substances. Initially, the ERLs applying in the Netherlands were: serious risk (SR), maximum permissible risk (MPR), and negligible risk (NR). However, the term "risk" is too strongly associated with the derivation of risk limits for single substances based on simple toxicity tests. The following new terms are, therefore, proposed:

- negligible effect (NE);
- maximum permissible effect (MPE); and
- serious effect (SE).

The criteria for water and sediment (i.e. the details of the metric) are set out below for both *in vivo* and *in vitro* bioassays. A schematic representation of the metrics is shown in Figure 19.1.

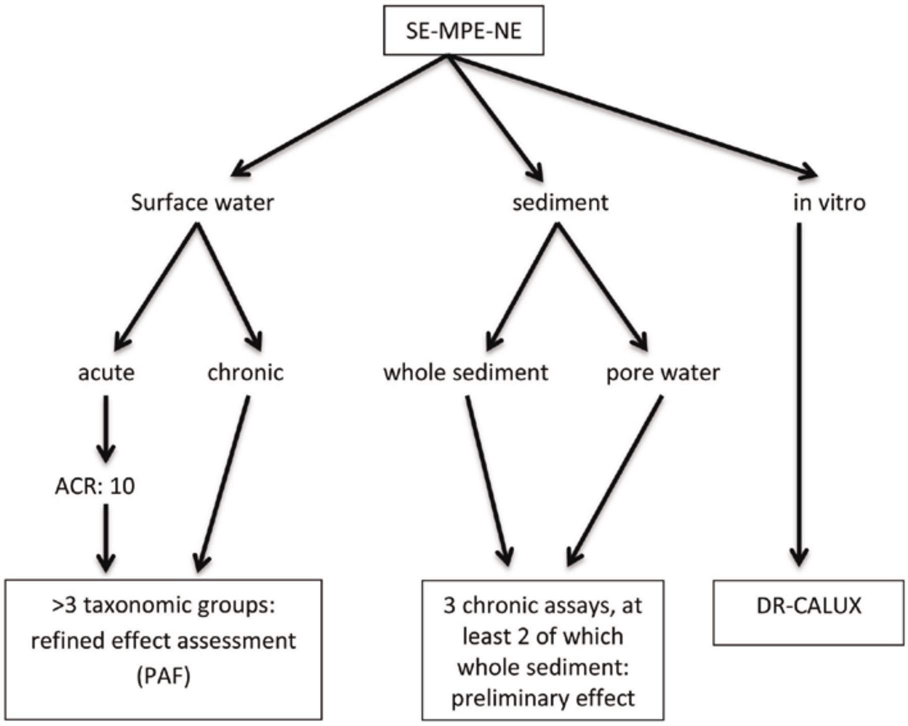


Figure 19.1. Summary of the metrics based on *in vivo* bioassays for surface water (and sediment), and on *in vitro* bioassays. NE, negligible effect; MPE, maximum permissible effect; SE, serious effect; ACR, acute–chronic ratio; PAF, potentially affected fraction (from Maas et al, 2003).

19.12 Proposed metric and criteria for use in *in vivo* bioassays

For the scaling of the results of these bioassays, a metric consistent with the NR–MPR–SR concept has been chosen: the NE–MPE–SE metric. Two points should be noted, however, regarding consistency with standards for individual substances. First, concerning the method, the same methods have been used for the metric as for substance standards, as described in the RIVM report on Guidance Document on Deriving Environmental Risk Limits (Traas, 2001):

- If NOEC values are present for four or more taxonomic groups, refined effect assessment is used. This uses species-sensitivity distributions (SSDs) based on the method according to Aldenberg and Jaworska (2000). The criterion for the MPR (or MPE in this case) is the 95% protection level, or PAF_5 (PAF = potentially affected fraction).
- If this condition is not met, preliminary effect assessment is performed, using "assessment factors". These factors range from 10 to 1000, depending on the nature of the study (acute or chronic) and the number of ecotoxicity data.

The same methods are thus used in the metric for bioassays proposed here, the actual choice of method depending on the number of chronic data available. It should be noted that the assessment factors for the preliminary-effect assessment are applied differently in the metric, although the principle is the same.

Second, as regards the factor for MPE/SE, a factor of 100 is used to derive the SR for individual substances from the MPR. This factor was chosen because many substances are often found together in the environment, and it takes account of the possible effects of combined toxicity (INS Steering Group, 1999). In bioassays, where samples from the field are used, this effect has already been taken into account, and a factor of 10 can be used for converting MPE to SE.

There are also a number of essential differences between *in vivo* bioassays with aquatic organisms and with sediment dwellers, which have implications for the metric:

- in sediment, unlike in freshwater, it is virtually only possible to use *chronic* tests;
- it is possible to use dilutions for both surface water and sediment, based on the undiluted or untreated sample (the ″as is″ sample). However, unlike sediment, a water sample can be concentrated, for example, with a 1 : 1 mix of XAD-4 and XAD-8 (de Zwart and Sterkenburg, 2002). Using this technique on water samples makes it easier to scale up the results of *in vivo* bioassays using aquatic organisms to the ″full″ metric NE–MPE–SE (including SE).

19.13 Standard for *in vivo* bioassays for surface water

19.13.1 Method 1: Standard with ″preliminary effect assessment″

Three acute or chronic tests from different taxonomic groups are applied as shown in Table 19.3.

Table 19.3. Details of the metrics for surface water

Acute tests	
NE (negligible effect)	in 3 acute tests effect = 0 (in practice < EC_{50}), Cf = 100
MPE (maximum permissible effect)	in 3 acute tests effect = 0 (in practice < EC_{50}), Cf = 10
SE (serious effect)	in 1 acute test effect ≥ EC_{50}, Cf = 10 or in 2 acute tests EC_{20} < effect < EC_{50}, Cf = 10
Chronic tests	
NE (negligible effect)	in 3 chronic tests effect = 0, Cf = 10
MPE (maximum permissible effect)	in 3 chronic tests effect = 0, Cf = 1
SE (serious effect)	in 1 chronic test effect ≥ EC_{50}, Cf = 1 or in 2 chronic tests NOEC < effect < EC_{50}, Cf = 1

EC_{50}, mean effective concentration, produces a 50% effect in the bioassay; NOEC, no observed effect concentration; Cf, concentration factor compared with the untreated sample (original water sample) (from Maas et al., 2003).

19.13.2 Method 2: Standard with "refined effect assessment" (PAF approach; see Figure 19.2)

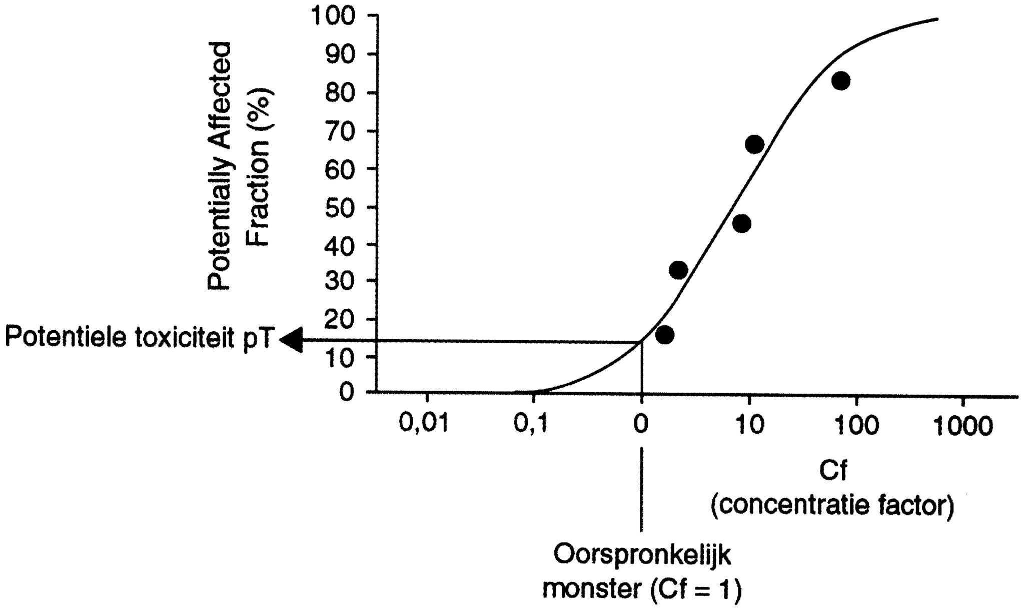


Figure 19.2 Use of a response curve to estimate the potentially affected fraction (PAF %) (Oorspronkelijk monster = original sample) (from Maas et al., 2003).

The method works as follows:

- At least four chronic values for different taxonomic groups must be available.
- Both acute and chronic bioassays can be used.
- Results of acute tests are expressed as the concentration factor necessary to reach a 50% effect in the bioassay. These results are transformed into a chronic value by applying an acute–chronic ratio (ACR) of 10 (de Zwart and Sterkenburg, 2002).
- For chronic values, a species-sensitivity distribution is assessed following a log-logistic distribution (Traas, 2001).
- The extent to which the PAF_5 (for the MPE) and PAF_{50} (for the SE) are exceeded in the undiluted Cf = 1 sample is determined.

In order to determine the NE, the Cf [associated with the MPE (PAF_5)] is defined and divided by 10. This gives the Cf at which the NE acts. This result is compared with the results of the undiluted sample in order to determine whether this conforms to the MPE or the NE.

The MPE on the metric for surface water thus corresponds to the level at which no effect is measured in three *chronic* tests with different taxonomic groups on the "as is" sample (Cf = 1). On the basis of three *acute* tests, the MPE corresponds to the level at which no effect (in practice $<EC_{50}$) is measured when the sample is concentrated by a factor of 10 (Cf = 10) relative to the "as is" sample. This factor 10 is based on the ACR of 10 (see above).

The above presentation of a metric for *in vivo* bioassays in surface water states no preference for the use of acute or chronic bioassays. A metric has been developed for both types (Table 19.3). The choice of chronic or acute will depend partly on the specific circumstances at the locations studied (e.g. the compartment to be assessed, knowledge of the degree of pollution). A choice will, therefore, have to be made for each type of study and compartment. In this choice, the advantages of acute tests will often outweigh the drawbacks. For instance, chronic effects are sometimes difficult to observe even in concentrates. It is easier to conduct several acute tests simultaneously. Furthermore, the shorter duration of acute tests means the composition of the matrix (water) is more constant, an issue that has proven problematic in chronic tests. If the choice of more acute tests or more chronic tests depends on cost, in our experience, the first option is generally preferred (more acute tests, with other organisms or other taxonomic groups).

It is possible to illustrate how the metric for surface waters works in practice on the basis of a 1996 study of the toxicity of surface water in Dutch waters at 15 locations (de Zwart and Sterkenburg, 2002). Acute toxicity tests were performed with five *in vivo* bioassays: the Microtox assay, an algal photosynthesis test using *Selenastrum capricornutum*, the Rotox test, the Thanmotox test, and the Daphnia IQ test. A PAF curve was fitted after the acute EC_{50} values were extrapolated to chronic NOEC values with a factor of 10. Although de Zwart and Sterkenburg (2002) estimated the toxicity of the original water sample using the pT method (pT: toxic potency, or the PAF of the undiluted water sample), it is also possible to deduce from their results whether or not the MPE or SE was exceeded.

Another example of toxicity-based assessment is illustrated in Table 19.4. Water samples from the surface-water monitoring programme of the Western Scheldt estuary (NL) in the period 2000–2005 were extracted using the XAD extraction method (de Zwart and Sterkenburg, 2002). This is necessary to achieve an extract in which acute toxicity can be measured. The matrix of the samples is displaced by a standardized medium. Noise effects from, for instance, nutrients or salt concentrations are removed in order to decrease the number of false positive effects. The extracts were assayed with three different bioassays.

To interpret the test results, it is important to set criteria for acceptable effects in the undisturbed sample. Table 19.4 shows the results of a preliminary effect assessment using the test results of the three bioassays.

Table 19.4. Indication of toxicity in surface water of the Western Scheldt estuary on the basis of three different bioassay responses allowing a preliminary effect assessment, as proposed in Maas *et al.* (2003)

		C_F (ECF_{50})*			C_F (MTE)
Location	Date	*Daphnia*	Algae	Microtox	(from PAF_5)
SvOD-1	1-2-00	42	20	19	
SvOD-2	9-4-00	28	16	24	
SvOD-3	11-6-00	54	2.4	23	
SvOD-4	2-8-00	56	3.5	35	
SvOD-5	17-10-00	96	4.5	62	
SvOD-6	15-12-00	87	9	31	
SvOD-1	13-01-05	95	20	27	
SvOD-2	9-03-05	87	30	29	
SvOD-3	2-05-05	127	17	43	
SvOD-4	27-6-05	197	14	44	
SvOD-5	23-8-05	251	10	38	
SvOD-6	19-10-05	94	12	70	

W. Scheldt Vlissingen	4-6-03	416	52	15	2.0
W. Scheldt Honte	4-6-03	180	56	38	3.2
W. Scheldt Terneuzen	4-6-03	403	28	57	4.0
W. Scheldt Hansweert	2-6-03	243	16	84	17.2
W. Scheldt Boei s.v WO3	2-6-03	271	15	97	3.2
Scheldt Bath	3-6-03	271	9	52	1.8
Schaar vo Doel (SvoD)	3-6-03	92	9	50	1.6
Scheldt Antwerpen	18-6-03	144	2	23	0.4

*Corrected for recovery

Expected chronic effect in surface water:

green = negligible effect (NE)

yellow = NE < effect < maximum permissible effect (MPE)

red = serious effect (SE)

19.14 Experience in the UK

The oyster embryo bioassay has been used widely for the measurement of water quality. Surveys in the early 1990s showed no adverse water quality offshore and occasional instances of poor water quality in some UK estuaries. Recent surveys have only been conducted in estuaries. The range of response measured is per cent net response (PNR); values range from 0 to 100, where 100 indicates that no oyster embryos developed. A value of ≥20 PNR is regarded as an adverse, but negligible effect, a value of between 50 and 80 is cause for concern (maximum permissible effect), and >80 is a serious effect. PNR values between 20 and 50 have been measured in some UK estuaries, but repeated sampling has shown the poor water quality to be transitory.

Over the past six years, trials have been conducted using water extraction techniques. Initially, these were conducted using a hexane liquid–liquid extraction technique (Thain and Kirby, 1996). More recently, SPMD extraction procedures have been used successfully (K. V. Thomas *et al.*, 1999, 2000), and we have developed a battery of bioassay tools to use, including bivalve embryo development, *Tisbe* bioassay, echinoderm larval development, fish embryo survival, phytoplankton growth and a number of *in vitro* bioassays, YES and YAS oestrogen screen, and the aryl hydrocarbon receptor (AhR)-based assay. The data have not yet been published, but assessment of the water quality results show that contaminant concentration factors (CCF, i.e. the concentration of the contaminants in a water sample required to elicit an EC_{50}) are generally:

- >1000 at distant offshore stations such as the ICES Reference Stations;
- 500–1000 at offshore stations such as the western English Channel;
- 200–500 at intermediate stations;
- 50–200 at inshore stations;
- 10–50 at coastal stations and estuaries;
- >10 only observed in estuaries.

The use of these bioassays and water concentration techniques is in development and, therefore, no assessment framework has been established. However, it is clear that the procedures permit water quality to be assessed and mapped, but this has to be interpreted within the limitations and restrictions of the chemical process.

19.15 Conclusions

Water *in vivo* bioassays are available for immediate deployment within the OSPAR JAMP CEMP. These bioassays have been recommended by ICES and are of sufficient standing in terms of methodological development, ease of use, and application for uptake across the whole OSPAR area. Quality assurance procedures are in place for most of the bioassays, and are provided for by BEQUALM. Therefore, bioassay data can be submitted to the ICES database for subsequent assessment as appropriate by ICES/OSPAR.

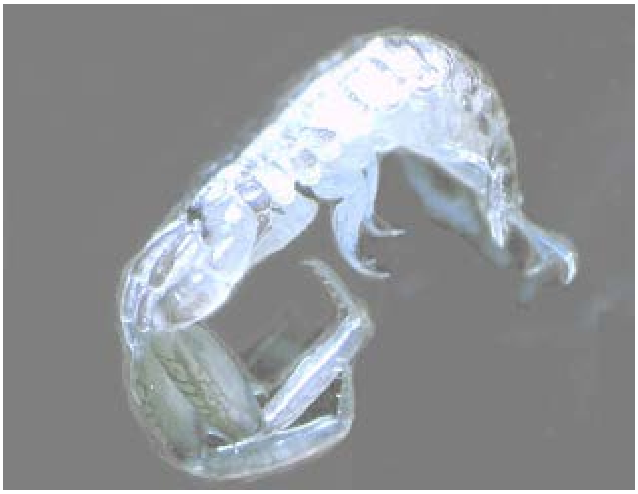
Bioassays should be deployed as a "battery of tests" and should include a minimum basic set, possibly of three or more. However, the composition of what the set needs to comprise requires further work. The range of bioassays needs to be expanded to include all trophic levels and phyla such as echinoderms.

The sampling strategy and design of water quality monitoring for spatial and temporal monitoring purposes needs to be clearly defined, particularly the role of water concentrates. In this respect, there is an important need to validate appropriate protocols for extraction methods and subsequent *in vivo* and *in vitro* testing.

Background response levels and assessment criteria for water bioassays currently in JAMP are available.

20 Background document: whole-sediment bioassays with amphipods (*Corophium* spp.) and *Arenicola marina*

Ricardo Beiras, John Thain, and Dick Vethaak



20.1 Background

The toxicity of sediment can be assessed either through the exposure of pelagic organisms to sediment seawater elutriates or to pore waters, or through the exposure of test organisms to whole sediment. The *Rhepoxynius abronius* amphipod test is commonly used in North America to evaluate the quality of whole sediments intended for dredging or dumping, and very detailed protocols are available (Swartz *et al.*, 1985; ASTM, 1992). The endpoint is survival after incubation for 10 d in the whole sediment at 20°C. These protocols can be easily adapted to the European species *Corophium* spp. Some efforts have already been made to compare methods and sensitivities for different amphipod species (van den Hurk *et al.*, 1992; Casado-Martínez *et al.*, 2006).

The *Corophium* genus is broadly distributed across Europe. An internationally agreed protocol for toxicity testing of offshore chemicals with *C. volutator* has been published (OSPAR, 1995). ICES has also provided detailed methods (Roddie and Thain, 2001). Those protocols are also suitable for other macroscopically indistinguishable *Corophium* species more abundant in southern Europe (*C. multisetosum*). In fact, the procedure can be used not only with any *Corophium* species, but with any infaunal amphipod (Roddie and Thain, 2001).

Other sediment-dwelling species from different taxa (polychaetes, echinoderms, bivalves) may also be suitable after methodological standardization and sensitivity comparisons with amphipods. Furthermore, *Corophium* is not tolerant of coarse-grain sediments. Should sandy sediments be tested, alternative species such as *Arenicola*, *Echinocardium*, or *Cerastoderma* will be needed. *Arenicola marina*, a direct-deposit-feeder, is widely distributed in European coastal waters and on the east coast of North America. The method has been tested nationally in the UK as well as in ring-tests under the Paris Commission and published by ICES (Thain and Bifield, 2001). It is suitable for bioassays on field-collected sediments and also for toxicity testing.

Some sublethal responses have been proposed as additional endpoints in order to enhance sensitivity, including reburial after the 10-d exposure (Bat and Raffaelli, 1998), and 28-d growth (Nipper and Roper, 1995). The later considerably delays the outcome of the test and may be a limitation for routine application. The use of fast-growing juvenile stages might overcome this limitation. In the test described by Thain and Bifield (2001), the bioassay endpoints include both mortality and a non-lethal indication of effect (inhibition of casting). The latter is monitored daily and can increase the sensitivity of the bioassay by an order of magnitude.

20.2 Confounding factors

The presence of toxic reduced compounds, such as unionized ammonia and H_2S, in interstitial and overlying water has been identified as a confounding factor in whole-sediment toxicity testing (Phillips *et al*., 1997). Grain size also affects amphipod survival (de Witt *et al*., 1988). These studies have been carried out with North America species. Further research on this topic with *Corophium* spp. is needed.

The size of the animals is standardized for the methods, and adherence to the recommendations will ensure that data are comparable. Also, control mortality can vary and be more of an issue at certain times of the year, but strict validity criteria for control mortality are provided.

20.3 Assessment criteria

According to the US EPA (1998), a sediment sample is classified as toxic when it induces an amphipod mortality 20% higher than control, and the difference is statistically significant. Similarly, ICES (2008b) currently recommends classification as "elevated" when *Corophium* mortality is >30% and "high concern" when it is >60%. For *Arenicola*, these benchmarks go down to >10% for "elevated" and > 50% for "high concern" (ICES, 2008b).

ANOVA and *a posteriori* Dunnett's test allow comparison and classification of sampling sites into homogeneous groups according to their toxicity. Mortality data must be arcsine-transformed prior to analysis. When data from different test rounds were pooled together for statistical analysis, mortalities must be previously corrected by the respective controls using Abbott's formula: $P' = (P–P_c) \times 100/(100–P_c)$; where P and P' are the raw and corrected mortality percentages, and P_c is the control mortality. In this case, no control treatment is available, and Tukey's rather than Dunnett's *post hoc* test is preferred. Again, mortality data must be arcsine-transformed prior to analysis.

20.4 Quality assurance

Sediment manipulations during sampling, storage, and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended for toxicity testing should not be frozen, but should be stored under refrigeration in the dark inside airtight containers and tested within one week.

With regard to the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. The following issues have been identified as relevant to the success of the intercalibration round. Sediment samples should be homogeneous in grain size and organic content, but ranging from pristine to highly polluted. Preservation of the sediment from sampling to testing should be similar for all participants, including time and temperature. Because this species has no commercial value, the test individuals must be collected from the field and acclimated and maintained in the laboratory long enough to assess the population health prior to testing.

Acceptability criteria must be developed with regard to minimum survival/reburial in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and the interpopulation variability. Stringent acceptability criteria are essential to guarantee reliable toxicity data, particularly when test organisms come from wild populations and experience a sharp change in environmental conditions in the laboratory. In an intercalibration round in Spain, Casado-Martínez *et al*. (2006) set acceptable maximum control mortality at 10%, following US EPA (1994). Roddie and Thain (2001) raised this threshold to 15%. Results of background response levels for *Corophium* and *Arenicola* bioassays are shown in Table 20.1. All laboratories show a 90th percentile for mortality higher than 10%, and most are above the recommended 15%, indicating that special care must be taken to avoid any damage to the individuals during collection, maintenance, and transfer into experimental beakers.

The third year of a bioassay programme ran within BEQUALM from December 2006 to June 2007; this included the 10-d *Corophium volutator* survival bioassay.

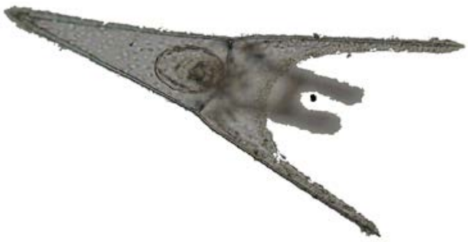
The background response values for *Arenicola marina* are based on extensive surveys in the UK: 63 stations around the UK (Thain and Bifield, 2001) and estuarine surveys conducted over several years (UK Charting Progress, 2010).

Table 20.1. Background response levels for whole-sediment bioassays (mortality); the median 90th percentile (i.e. BAC) is 18.4%

Test	Laboratory	Average	10th percentile	Median	90th percentile	*n*
Corophium	RIKZ	12.3	6.6	10.5	19.3	4
Corophium	Cefas	9.5	0.0	6.7	20.0	21
Corophium	IEOV	7.7	5.6	6.3	10.8	5
Corophium	AZTI	10.4	4.8	10.8	17.4	27
Arenicola	Cefas	4.7	0.0	0.0	13.3	20

21 Background document: seawater, sediment elutriate, and pore-water bioassays with early developmental stages of marine invertebrates

Ricardo Beiras, Dick Vethaak, and John Thain



BIVALVE D-LARVA SEA URCHIN PLUTEUS LARVA

21.1 Introduction

The embryogenesis and early larval development of marine invertebrates have been frequently used as a rapid, sensitive, cost-effective biological tool for the assessment of seawater, sediment elutriates, and pore-water quality. Early developmental stages are generally more sensitive than adults and the weakest link in the life cycle. The embryo–larval bioassays detect a broad spectrum of toxicants at comparatively low concentrations in the order of 1 $\mu g\ l^{-1}$ for TBT and other antifouling products; 10 $\mu g\ l^{-1}$ for Hg, Cu, and Zn; 100 $\mu g\ l^{-1}$ for Pb, Cd, and other metals; 1 $mg\ l^{-1}$ for organochlorine pesticides, detergents, and refined oil; and 10 $mg\ l^{-1}$ for crude oil (Kobayashi, 1995; His *et al*., 1999).

Detailed descriptions of methods and applications are available for bivalves (Woelke, 1961; Thain, 1991; His *et al*., 1999) and sea urchins (ASTM, 1995; Carr, 1998; Saco-Álvarez *et al*., 2010). Gametes are obtained from mature adults either by stripping or thermally induced spawning, fertilized *in vitro* in a measuring cylinder, and delivered into the experimental samples. After 24–48 h incubation at 18–24°C (depending on the species), samples are fixed and microscopically observed to record the percentage of normally developed larvae and, for sea urchins, size increase.

Sensitivity of embryos of different species to the main pollutants of concern in the marine environment is very similar, particularly within bivalves. This allows comparison of results of embryo–larval bioassays conducted with different species. A review on the EC_{50} values of 18 priority pollutants to bivalve vs. sea urchin embryos reflected a correlation coefficient $r^2 = 0.96$ ($p < 0.01$) and a slope $b = 1.00$ (Beiras and Bellas, 2008). Owing to their abundance and broad geographical distribution or availability from commercial sources, the following species are recommended: *Crassostrea gigas*, *Mytilus edulis/galloprovincialis*, *Paracentrotus lividus*. For sea urchins, other species like *Strongylocentrotus droebachiensis* and *Echinus sculentus*, extend the applicability of the assay with indigenous species to northern countries (Figure 21.1).

Within bivalves, *Crassostrea gigas* and, in the US *C. virginica*, oysters have been most often used for embryo–larval ecotoxicological bioassays because, unlike the mussel or the native flat oyster (*Ostrea edulis*), fertile gametes in *Crassostrea* can be obtained straight from the gonad by stripping, although this method requires high percentages of embryogenesis success in the controls to guarantee comparability of the results (His *et al.*, 1999). The marine mussels of the *Mytilus* genus occurring in European waters (*M. edulis* and *M. galloprovincialis*) are nearly ubiquitous, easy to collect, and easy to maintain in aquaria. These species also have the advantage that the adults are commonly used in marine pollution monitoring programmes, and OSPAR encourages the use of the same species for different biological tools of pollution assessment, spanning molecular, cellular, and individual responses. Another advantage of the mussel embryogenesis bioassay is that this species is tolerant of a broader range of salinities, including estuarine waters down to 20 ppt (His and Beiras, 1995). The *Paracentrotus lividus* sea urchin has a somewhat more restricted distribution, but it is easier than bivalves to feed and maintain in captivity, thus avoiding accidental spawning. Another advantage of the sea urchin embryogenesis bioassay is that it provides a quantitative, more gradual, observer-independent, and statistically treatable response: size increase (Saco-Álvarez *et al.*, 2010).

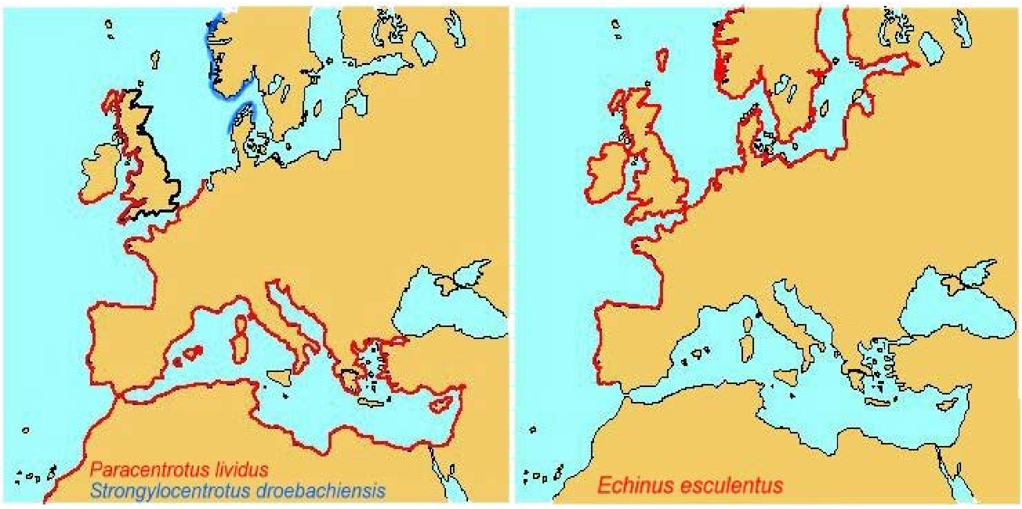


Figure 21.1. Geographical distribution of *Paracentrotus lividus*, *Strongylocentrotus droebachiensis*, and *Echinus esculentus* along European coastlines.

Currently, the main limitation of the embryo–larval bioassays is the availability of reliable, good-quality biological material year-round, particularly outside the natural spawning season of the different species, which changes among different European countries. The maintenance of fertile adult stocks in aquaria is feasible, particularly for sea urchins, and conditioned bivalves should be available from aquaculture facilities, but even commercial hatcheries are unable to provide 100% reliable adult broodstocks year-round. Cryopreservation of gametes of bivalves and sea urchins is a promising solution to provide homogeneous biological material at any time, but these techniques are currently still under development, and standard methods are not available. Combination of different species with different spawning seasons still seems to be necessary.

The toxicity of sediment can be assessed by either obtaining an elutriate from the sediment (by mixing with control seawater) or by directly obtaining the interstitial pore water from the sediment and undertaking toxicity tests on these aqueous solutions using water-column (pelagic) organisms. The advantages of the first method are: (i) smaller amounts of sediment and simpler equipment are necessary;

and (ii) the environmental parameters of the elutriate (dissolved oxygen, pH, salinity, ammonia, sulfides) are closer to those of the natural water column than for pore water, in particular when dealing with anoxic or hypoxic sediments. These parameters are the most common source of false positives (see confounding factors), and pore water requires adjusting their values within the optimum range for the test species prior to testing. In reverse, pore water has the advantage that no control seawater is needed and the dilution of the potential toxicants present is lower, enhancing sensitivity. The choice of the method can depend on sampling constrictions and sample availability, because when the confounding factors are taken into account, both methods yield comparable results (Beiras, 2001).

In general, the embryo–larval bioassay showed higher sensitivity than the amphipod bioassay to polluted sediments (Becker *et al.*, 1990; Long *et al.*, 1990; Carr and Chapman, 1992), although similar sensitivities have also been reported (Williams *et al.*, 1986). However, the differences in estimates of toxicity using different organisms can be large, and different tests may reflect different patterns or mechanisms of toxicity (Long *et al.*, 1996). Therefore, comparisons of different sediment toxicity tests must be conducted using samples representing a broad range of types of pollution in order to evaluate the comparability of the different tests.

21.2 Confounding factors

In order to avoid false positives, water quality values in the elutriate (or pore water) must be checked prior to testing and must fall within optimum ranges for the embryo development of the test species, or otherwise they must be adjusted. For molluscs, His *et al.* (1999) provide a broad review on this topic. Generally speaking, full salinity, a pH >7.5, and a dissolved oxygen concentration >2 mg l^{-1} are required. This is particularly important for pore waters from highly reduced sediments, which broadly depart from those values. For sea urchins, Saco-Álvarez *et al.* (2010) gave an optimal range for salinity of from 31 to 35, and from 7.0 to 8.5 for pH.

The presence of toxic substances such as unionized ammonia and H_2S has been identified as the main sources of false positives in sediment elutriate toxicity testing, where the objective is to investigate responses to chemical contaminants (Cardwell *et al.*, 1976; Matthiessen *et al.*, 1998b). Some threshold toxicity values for sea urchin and bivalve embryos are available in the literature (Knezovich *et al.*, 1996), but further research is strongly needed on this topic. For NH_3, Saco-Álvarez *et al.* (2010) obtained an EC_{10} of 68.4 μg l^{-1} and a NOEC/LOEC of 40/80 μg l^{-1} using *Paracentrotus lividus*.

With regard to temperature, elutriates and pore waters are microbially rich, and exposure to high temperatures during manipulation should be avoided. This includes centrifugation, when necessary. For incubation, 20°C (48 h) is recommended for mussels and *Paracentrotus lividus* urchins, and 24°C (24 h) for *Crassostrea gigas* oysters.

21.3 Ecological relevance

Ecological relevance is one of the strong points of the embryo–larval bioassay. Any impairment of embryo development would lead to reduced recruitment and decrease population size.

21.4 Assessment criteria

Marine invertebrate embryo–larval bioassays have resorted to different species and a suit of endpoints. This issue needs to be discussed prior to the implementation of assessment criteria.

21.5 Endpoints measured

The endpoint recorded in the standard embryo–larval bioassays is the percentage of morphologically normal larvae. The definition of morphological abnormalities varies among authors and, obviously, among test species. For the sake of routine applicability it is advised that only very conspicuous abnormalities are taken into account. This would reduce the time necessary to record the endpoint and facilitate automatization and observer independence. In bivalves, normal D-shape is advised as a normality criteria. This excludes larvae with protruding mantle and convex hinge. Illustrations of these abnormalities can be found in Quiniou *et al*. (2005). However, more detailed abnormalities, such as the presence of indentations in the larval shell, would complicate observation and, in our view, should not be taken into account at this stage, but may be considered as a field for future research.

In sea urchins, normal larvae should exhibit four fully formed arms (two longer post-oral arms and two shorter oral arms) and a regular outer contour of the body. Prepluteus stages, where oral arms are not yet fully separated, or larvae with missing arms should be considered as abnormal. However, identification of more detailed abnormalities, such as those related to the internal anatomy of the larvae (skeletal rods, gut), would greatly complicate observation. These even depend on the position of the larva under the microscope.

An alternative endpoint for the sea urchin test—measurement of the size increase in 48 h—was recently proposed by Saco-Álvarez *et al*. (2010). This avoids lengthy and subjective microscopical inspection, speeds up test readings, makes automatic reading feasible, and allows a more than twofold increase in sensitivity compared with the classical morphological endpoint.

21.6 Assessment criteria

21.6.1 Discrete approach

ICES (2008b) currently recommends classification of the toxicity of a liquid sample as "elevated" when embryo abnormalities are >20% for bivalves and >10% for sea urchins, and "high concern" when they are >50% for both invertebrates.

Generally speaking, an elutriate can be classified as toxic when it induces a statistically significant reduction in the endpoint (either normal morphology or size increase) compared with the elutriate from the reference site, for a confidence level of 95%. Percentages of response must be arcsine-transformed prior to analysis using ANOVA and *a posteriori* Dunnett's test, comparing each sampling site with the reference site. The difficulty here is to establish a reference site, based on comprehensive analytical data, that is not polluted, but is otherwise similar to the problem sites (see confounding factors). Control seawater may not be appropriate as a reference because it lacks the physico-chemical and microbiological properties of an elutriate, some of which may affect the response.

21.6.2 Continuous approach

Once identified as polluted, the toxicity of any sediment elutriate that causes a marked inhibition in normal development can be quantified by serial dilution with reference seawater, and calculation of the toxic units (TU = 100/ED_{50}, where ED_{50} is the theoretical dilution, expressed in percentage, that causes 50% abnormal larvae). This parameter can be obtained by fitting the data for the serial dilutions to standard toxicity curves (logit, probit, etc.). When data from different campaigns are pooled together for statistical analysis, they must be previously corrected by the respective controls by using Abbott's formula: $P' = (P - P_c) \times 100/(100 - P_c)$, where P and P' are the raw and corrected abnormality percentages, and P_c is the control abnormality. Once corrected, percentages must be arcsine-transformed for subsequent analysis. When using this quantitative approach with sea urchins, larval length after 48 h, or even better, size increase from fertilized egg after 24 h, is preferred to percentage of normal larvae. This is because size increase is a more sensitive, and thus more discriminant, response than morphologically normal development (Saco-Álvarez *et al.*, 2010).

For the sea urchin test, Durán and Beiras (2010) developed quantitative assessment criteria for the size-increase endpoint on the basis of the distribution of results from sites not significantly different from the reference. The methodology to obtain BAC and EAC values followed OSPAR (2009). The resulting BAC value was per cent net response (PNR) = 0.702, which means a 30% decrease in growth (size increase) in the tested population.

Using different percentiles of these distributions, assessment criteria for PNR and TU data were obtained (Table 21.1). A BAC of 32 was set for mussel larvae. EAC values of 50% were retained for both mortality (mussel embryo) or reduced growth (sea urchin embryo, as recommended earlier by ICES.

Table 21.1. Background response for mussel embryo bioassays (mortality) (data from the Spanish Institute of Oceanography)

AVERAGE	90TH PERCENTILE	MEDIAN	10TH PERCENTILE	*n*
14.7	32.3	8	3.2	38

21.7 Quality assurance

Sediment manipulations during sampling, storage, and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended for toxicity testing should not be frozen, but should be stored under refrigeration in the dark inside airtight containers and tested within one week. Some authors argue that testing can be delayed by freezing the liquid phase (elutriate or pore water) after elimination of particles. However, it must be taken into account that glassfibre filters adsorb metals, and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken, salinity checked, and adjusted, if necessary.

Concerning the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. In these intercalibrations, it would be desirable that not only different populations of a certain species, but also different species (oysters, mussels, clams, sea urchins) were included.

The control treatment in an embryo–larval bioassay gives essential information regarding biological quality of the test organisms. Acceptability criteria must be developed concerning minimum embryogenesis success and larval length in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and the interpopulation variability. For bivalves, His *et al*. (1997) reported mean values in controls ranging from 75.8 to 97.0, thus suggesting a minimum of 75% normality, whereas Quiniou *et al*. (2005) arbitrarily recommend a minimum of 80% normal D-larvae in the control as acceptability criterion (see also AFNOR, 2009). Preliminary results of background response levels for *Mytilus* embryo bioassays are shown in Table 21.1. Taking as acceptability criteria the 10th percentile of the distribution of all controls with natural filtered seawater (FSW) throughout several years during the natural spawning season (April, May, and June), a minimum of 68% normal D-larvae in controls is required. Nevertheless, if the bioassay is carried out outside the spawning season, failure to reach the acceptability criteria is likely to occur, and a compromise between sensitivity and feasibility must be reached.

For *P. lividus* normal larval development, the distribution of the endpoints measured (percentage of normal larvae and size increase) in controls with natural FSW and artificial seawater (ASW) over several years of tests conducted at 20°C for 48 h, is as shown in Figure 21.2 (Saco-Álvarez *et al*., 2010).

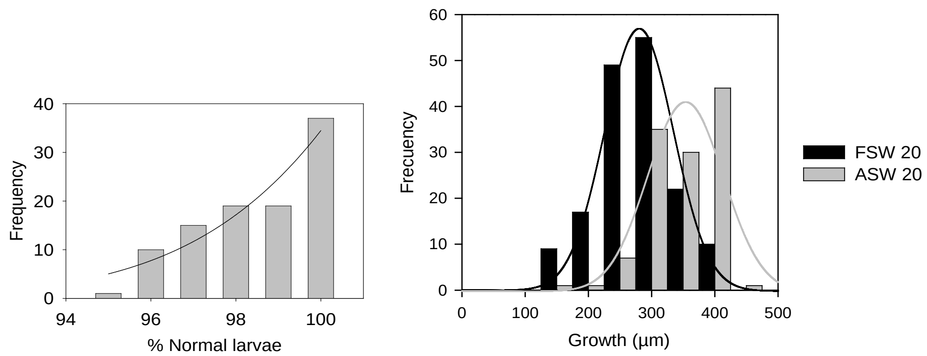


Figure 21.2. Distribution of endpoints for *P. lividus* normal larval development (percentage of normal larvae and size increase) in controls with natural filtered seawater (FSW) and artificial seawater (ASW) over several years of tests conducted at 20°C for 48 h (Saco-Álvarez *et al*., 2010).

From these data, and taking the fifth percentile as the acceptability criteria, a test is correct when mean response in the control exceeds 91% embryogenesis success and 218 µm size increase in FSW or 253 µm in ASW.

Percentage fertilization prior to testing must always be recorded. To run a reference toxicant test, it may be further useful to check the biological quality of the test organisms using a chart of the reference toxicant EC_{50} historical values.

22 Background document: sediment, seawater elutriate, and pore-water bioassays with copepods (*Tisbe*, *Acartia*), mysids (*Siriella*, *Praunus*), and decapod larvae (*Palaemon*)

Ricardo Beiras, John Thain, and Dick Vethaak

Tisbe battagliai

Siriella armata

Palaemon elegans

22.1 Background

The toxicity of sediment can be assessed either through the exposure of test organisms to whole sediment, or through the exposure of pelagic organisms to sediment seawater elutriates or to pore waters. In tests with elutriates or pore waters, crustaceans, and particularly early life stages, have been found to be several orders of magnitude more sensitive to insecticides than echinoderms and mollusca (Ramamoorthy and Baddaloo, 1995; Bellas *et al*., 2005). Crustaceans are also particularly sensitive to cadmium (Mariño-Balsa *et al*., 2000) compared with other marine invertebrates. Therefore, when these contaminants are suspected, the inclusion of a crustacean test within the battery of bioassays is strongly recommended.

Acute static survival tests with benthic (*Tisbe battagliai*) and planktonic (*Acartia tonsa*) copepods have been proposed to assess the biological quality of sediment elutriates (Matthiessen *et al*., 1998b). Detailed methods are available (Hutchinson and Williams, 1989; UNEP, 1989). The endpoint recorded may be mortality or motility after incubation for 48–96 h in the test samples at 20°C and 16 h light 8 h dark photoperiod. *Tisbe battagliai* is an abundant component of meiobenthic fauna, whereas *Acartia* and other calanoid copepods are components of the holoplankton in Atlantic waters. Both are easy to feed on microalgae. Ovigerous females can be isolated and age-controlled cultures can be obtained from the eggs. A water bioassay programme is running within BEQUALM which includes the 48-h *Tisbe battagliai* acute test.

Mysids, particularly the American species *Mysidopsis bahia*, are recommended by US EPA as test organisms for estuarine and marine water toxicity tests (US EPA, 2002). The maintenance of fertile adult stocks in aquaria, fed on *Artemia*, is feasible. Because these organisms undergo direct development in short periods, they are suitable for life-cycle assessments. Some European mysids, such as *Neomysis* (for brackish waters), *Praunus* (Mclusky and Hagerman, 1987; Garnacho *et al*., 2000) and *Siriella* (Pérez and Beiras, 2010), have been proposed, but sensitivity intercomparisons are lacking. Also, the salinity range of tolerance for each species must be determined before recommendation for routine toxicity testing.

The use of decapod early life stages is less frequent (Cheung *et al*., 1997; Mariño-Balsa *et al*., 2000). The main advantages are the economic value of some species (shrimps, crabs) and the possibility of obtaining ovigerous females from commercial stocks.

The main difficulty is in finding broadly distributed species across all Europe. The *Palaemon* genus may be a potential candidate because it has a broad geographical distribution from the Mediterranean Sea to the North Sea, it is easy to feed, maintenance of fertile adult stocks in aquaria is feasible, and larval development is well known.

22.2 Confounding factors

In order to avoid false positives, water quality parameters in the elutriate (or pore water), specifically salinity, pH, and dissolved oxygen, must be checked prior to testing and must fall within optimum ranges for the survival and motility of the test species, or otherwise they must be adjusted. This is particularly important for pore waters from highly reduced sediments, which broadly depart from those values.

More often, the presence of toxic reduced compounds, such as unionized ammonia, and H_2S, have been identified as the main sources of false positives in sediment elutriate toxicity testing (Cheung *et al*., 1997). Further research is needed on this topic.

22.3 Ecological relevance

Copepods and mysids are dominant components of holoplankton in marine ecosystems. They are primary consumers and an important food source for fish. Therefore, any toxicant affecting them is a threat to the whole foodweb in coastal and oceanic ecosystems.

22.4 Assessment criteria

ICES (2008b) currently recommends classification of the toxicity of a seawater sample as "elevated" when *Tisbe* mortality is > 10% and "high concern" when it is > 50%.

22.5 Quality assurance

Sediment manipulations during sampling, storage, and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended for toxicity testing should not be frozen, but should be stored under refrigeration in the dark inside airtight containers, and tested within one week. Some authors argue that testing can be delayed by freezing the liquid phase (elutriate or pore water) after elimination of particles. However, it must be taken into account that glassfibre filters adsorb metals, and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken, salinity checked, and adjusted, if necessary.

Concerning the effect of homogeneous biological material, interlaboratory comparisons carried out following strict protocols are necessary. In these intercalibrations, it would be desirable that not only different populations of a certain species, but also different species (*Tisbe*, *Tigriopus*, *Acartia*, mysids, shrimp larvae) were included.

Acceptability criteria must be developed concerning minimum survival/motility in the control for a test to be considered reliable. Those criteria must take into account both the normal seasonal variability within a certain population and interpopulation variability. A stringent acceptability criterion is essential to guarantee reliable toxicity data, particularly when test organisms come from wild populations and experience a sharp change in environmental conditions in the laboratory, and protocols should include a period of acclimation to avoid sharp changes. Results of background response levels for *Tisbe* bioassays are shown in Table 22.1, resulting in a BAC of 5.0.

Table 22.1. Preliminary results of background response levels for *Tisbe* bioassays (mortality)—data from Cefas

AVERAGE	10TH PERCENTILE	MEDIAN	90TH PERCENTILE	*n*
1.3	0.0	0.0	5.0	28

Running a reference toxicant test may be a further useful check for the biological quality of the test organisms. The reference toxicant should ideally be stable in aqueous solution and not dangerous to human beings.

23 Technical annex: protocols for extraction, clean-up, and solvent-exchange methods for small-scale bioassays

Hans Klamer, Knut-Erik Tollefsen, Steven Brooks, and John Thain

23.1 Introduction

The aims of this report are to:

- produce standardized protocols for bioassay extractions;
- enhance consistency of applications between laboratories;
- ensure applicability throughout the OSPAR maritime area, including in estuarine waters; and
- ensure comparability of reported data for assessment purposes.

23.1.1 History

This report has been developed from a previous review and relates particularly to background documents on water and sediment bioassays and *in vitro* bioassays prepared by the ICES Working Group on Biological Effects of Contaminants (WGBEC) and ICES/OSPAR Study Group on the Integrated Monitoring of Chemicals and their Effects (SGIMC). This chapter describes a recommended methodology for extraction protocols for use in small-scale *in vitro* and *in vivo* bioassays

23.1.2 Scope

- This procedure will be used to provide samples for measurements of toxicity in environmental samples and assessment of their potential environmental risk. Other applicable approaches include toxicity identification evaluation (TIE)/effects-directed analysis (EDA), and toxicity tracking of effluent and produced-water discharges.
- Extraction of aqueous, solid, and fish bile samples.
- Preparation of extracts for *in vivo* bioassays including: mussel and oyster embryo, *Tisbe*, *Daphnia*, *Nitocra*, *Acartia*, sea urchin embryo, fish embryo, algal growth, algal PAM, and macrophyte germination.
- Preparation of extracts for *in vitro* bioassays (e.g. Microtox, Mutatox, YES, YAS, DR/ER/AR-CALUX, TTR, *umu*-C, Ames-II, fish cell lines).

23.2 Extraction protocols

In this chapter, extraction protocols will be presented covering a range of types of sample: solid, aqueous, or fish bile. Depending on the bioassay used, differences in extraction solvent and, in particular, sample clean-up may be applied.

Klamer *et al*. (2005a) proposed the following operational definitions of solid and aqueous samples:

- **solid samples:** particulate material, sediments, sludges, aerosols, suspended solids, and soils;
- **aqueous samples:** surface or deep waters, wastewater, sediment pore water, potable water, rain, snow, ice.

Before detailed protocols are presented, the basic layout of each extraction and clean-up protocol is given in Table 23.1.

Table 23.1. Protocol steps and associated comments for extraction of solid, aqueous, and file bile samples

Solid samples	
Protocol steps	**Comment**
1. Sample preparation	Sample sieved, when necessary (e.g. sediment), dried, and homogenized
2. Extraction of crude sample	Accelerated solvent extraction (ASE) or Soxhlet extraction. Solvents: dichloromethane (DCM) or hexane with methanol or acetone as modifier
3. Concentration of crude extract	Automatic (e.g. Turbovap or manual) concentration to smaller volume, typically less than 5 ml. Remove coextracted water if necessary
4. Clean-up of crude extract	Gel permeation chromatography (GPC) with DCM for broad-spectrum contaminant profiling. Reversed or normal phase HPLC for more selectivity. Sulfur removal may be necessary
5. Concentration of cleaned extract	Automatic (e.g. Turbovap or manual) concentration to smaller volume, typically less than 1 ml. Final test solvent (e.g. DMSO or methanol may be added as keeper)
Aqueous samples	
Protocol steps	**Comment**
1. Sample preparation	Sample filtered and/or pH-adjusted when necessary
2. Extraction of crude sample	Solid phase extraction (SPE) with resin (e.g. XAD) or cartridge-containing adsorbents (C8, C18, LiChrolut™, POCIS)
3. Concentration of crude extract	Automatic (e.g. Turbovap or manual) concentration to smaller volume, typically less than 5 ml
4. Clean-up of crude extract	GPC with DCM for broad-spectrum contaminant profiling. Reversed or normal-phase HPLC for more selectivity
5. Concentration of cleaned extract	Automatic (e.g. Turbovap or manual) concentration to smaller volume, typically less than 1 ml. Final test solvent (e.g. DMSO or methanol may be added as keeper)
Fish bile samples	
Protocol steps	**Comment**
1. Sample preparation	Thaw on ice
2. Pretreatment of crude sample	Deconjugation with a mixture of water, sodium acetate buffer and beta-glucuronidase–arylsulfatase. Total volume typically 1.5 ml
3. Extraction of pretreated sample	pH treatment with 100 µl 1N HCl, extraction with 2 ml ethyl acetate
4. Clean-up of crude extract	Precipitate any formed protein using isopropanol. Centrifugate. Repeat extraction
5. Concentration of extract	Manual concentration to dryness of combined ethyl acetate phases using N_2, solvent exchange into 50 µl DMSO

23.2.1 Protocol for extraction of dried, solid samples with accelerated solvent extraction (5–g sample)

Steps for dried solid (S) samples are as follows:

S.1) Assemble the ASE cells. Add a small layer of dried silica until cellulose filter is no longer visible.

S.2) Weigh approximately 5-g dried sample in the ASE cells (weighing accuracy mass ±0.1%).

S.3) Fill the ASE cells with dried silica and compact the content of the cells with the engraver pen. Close the cell and firmly twist the end-cap on the ASE cell.

S.4) Extract the sample using the following ASE settings:

Solvent	Pressure (psi)	Temperature (°C)	Preheat time (min)	Static time (min)	Flush volume (ml)	Purge time (s)	Static cycles
Hexane/Acetone 9:1 v:v	2 000	100	5	5	60	90	3
DCM or DCM/modifier[b]	2 000	45–100[a]	5	5	60	90	1–3[a]

[a]Set temperature to 45–50°C and number of cycles to 3 for use with ER-CALUX and similar tests.

[b]Methanol or acetone.

S.5) If water is coextracted, dry the extract using anhydrous sodium sulfate. Rinse with solvent. Evaporate the extract (until approximately 2–5 ml is left), in an automatic or manual set-up.

S.6) Proceed to solvent exchange or store the crude extract at –20°C until further use.

23.2.2 Protocol for extraction of aqueous samples with solid phase extraction devices

23.2.2.1 Extraction

Steps for aqueous (A) samples are as follows:

A.1) Assemble the SPE cartridge. For samples up to 20 litres, a single-column set-up is used. A Teflon tube is filled with glass wool to remove particulates and then the SPE columns are filled with methanol and attached in series with the C8 column first, followed by the ENV+. For 100-litre samples, a multicolumn system is used, where six Teflon tubes are set up as with the single-column system, but then attached to a manifold, allowing one sample to pass through all six columns simultaneously.

A.2) Set up the pressure system. From the pressure source, the air line passes through an air filter and then into a manifold. This allows for more than one vessel to be run at any given time, and also the airline diameter to be reduced. This line is then connected to the pressure vessel via a needle valve, ensuring the correct inlet/outlet is used (the inlet for the air is just a hole in the top of the vessel, the outlet has a pipe which goes to the bottom). From the outlet, another tube is connected which goes into the top of the single-column system or manifold for the multicolumn set-up.

A.3) Once the pressure lines are set up, the air line can be switched on, ensuring first that all needle valves are closed. The pressure should be no greater than 2 bar. The valve can then slowly be opened to allow a flow of approximately 40 ml min^{-1} through the columns.

A.4) Once all of the sample has passed through the column, allow the columns to dry by passing air through them. Label each column with sample site. Wrap in hexane-rinsed foil and store in a freezer at –20°C. Samples can be stored in the freezer for up to two months before elution.

23.2.2.2 Elution

A.5) Remove columns from the freezer and, while they are thawing, solvent rinse two glass sample collection tubes per column. Label the sample tubes.

A.6) In a fume cupboard, place the columns in the vacuum unit, with a Teflon tap. Fit a length of vacuum-proof hose to the unit, attaching the other end to a waste barrel. Another length of hose should run from the barrel to a vacuum pump.

A.7) Wash the columns with 10 ml RO or milliQ water. This will help to remove salt from saline samples.

A.8) Ensure columns are dry by sucking under vacuum for 10 min, or until there is no visible water dripping through the columns (whichever is longer).

A.9) Place a labelled collection tube under each column in a rack.

A.10) Elute each column with 10 ml DCM. Add 1 ml DCM to the column and allow to soak for 1 min with the tap closed. Open the tap and allow the solvent to drip through. Repeat this three times with 1, 4, and 4 ml DCM, respectively.

A.11) Remove the tube from under each column and replace it with a clean one. Repeat step A.7 with methanol.

A.12) Reduce the samples in volume to approximately 1 ml, and then combine the four fractions of each sample (C8 DCM, C8 methanol, ENV+ DCM, EMV+ methanol). For 100-litre samples, there will be six of each type of column. Combine all fractions.

A.13) There may be some water in the samples. This will form a layer or droplets in the DCM. If this is the case, take a glass column and packed with hexane washed anhydrous sodium sulfate. Add the samples to the top of the column. Elute with 5 ml DCM and collect in a labelled tube.

A.14) Blow down each extract to approximately 5 ml using, for example, a Turbovap at 30°C, 5 psi oxygen-free nitrogen. From this point, aliquots of samples can be solvent-exchanged into the appropriate solvent depending on the assay in question. Transfer sample into a glass ampoule. Store extracts in freezer at –20°C. Samples can be stored for a maximum of 1 year.

23.2.3 Protocol for extraction of fish bile samples

The extraction procedure described below for file bile (B) samples is taken from the work by Legler *et al*. (2002).

B.1) Thaw bile samples.

B.2) Transfer 100 μl of bile to glass test tubes.

B.3) Add 700 μl sodium acetate buffer (100 mm, pH 5.0 at 37°C), followed by 600 μl distilled water and 40 U of β-glucuronidase–arylsulfatase (from *H. pomatia*).

B.4) Incubate tubes overnight (17–18 h) in a water bath (37°C, gentle shaking).

23.3 Clean-up

23.3.1 Broad-spectrum clean-up

Clean-up procedures are applicable to all crude extracts. However, the user has to choose between two fundamentally different clean-up principles: *broad-spectrum* or *target* clean-up.

GPC, with DMC as eluting solvent, provides a sample with contaminants having a *broad spectrum* of physico-chemical properties. GPC separates on molecular volume and may, therefore, be used to easily remove, *inter alia*, humic acids and lipids. GPC column material, however, also has a secondary retention mechanism, based on electronic interaction between the column material and the extracted compound. This secondary mechanism is used for removal of molecular sulfur (as S8) from the crude extract, using DCM as eluting solvent. GPC clean-up requires careful calibration using a series of different compounds. This type of clean-up has successfully been applied to very different *in vitro* bioassays: Microtox, Mutatox, (anti)DR-CALUX, (anti)ER-CALUX, *umu*-C (e.g. by Houtman *et al*., 2004 and Klamer *et al*., 2005a).

C.1) Set-up of GPC equipment. For semi-preparative clean-up, large-diameter columns may be used in series, e.g. polystyrene-diphenylbenzene copolymer columns (PL-gel, 5 or 10 μm, 50 Å, 300 × 25 mm or 600 × 7.5 mm, preferably in a thermostatic housing at 18°C, with a PL-gel pre-column 5 or 10 μm, 50 × 7.5 mm). Use an HPLC pump with 10 ml min^{-1} dichloromethane as eluens.

C.2) Calibration. When necessary, determine the elution profile of individual compounds by injection of 2 ml of standard solutions (concentration 0.5–10 mg l^{-1}) and assessment of retention times at peak maximum and peak shape.

C.3) Set-up of the fraction collector. As a rule of thumb, the elution of parathion may be used to trigger the start of the collection of the cleaned sample, while the collection is stopped just before sulfur (as S8) elutes (elution of the extract is monitored using a UV detector at 254 nm). This range, however, should be carefully monitored using several reference compounds (in DCM solution). Examples of compounds that may be included in this mixture are: sulfur, pyrene, and ethyl-parathion. Depending on the particular application, other reference compounds may be needed (see e.g. Houtman *et al*., 2004).

C.4) Inject crude extract in batches of 200–2000 μl, depending on the capacity of the GPC column (semi-prep 25-mm column may be loaded with 2000 μl). Concentrate the collected sample fractions, proceed to solvent exchange or store at –20°C until further use.

23.3.2 Selective or dedicated clean-up

Selective clean-up using adsorption chromatography (e.g. reversed or normal phase liquid chromatography, with or without modifying additives like KOH, $AgNO_3$).

23.3.2.1 DR-CALUX

The clean-up of crude extracts for DR-CALUX measurements can be done with an acid silica column combined with TBA sulfur clean-up. The protocol for the DR-CALUX clean-up is as follows:

TBA sulfite solution

1. Wash a 250-ml separation funnel with hexane, fill the funnel with 100 ml HPLC water, and dissolve 3.39 g TBA.
2. Rinse the solution three times with 20 ml hexane.
3. Dissolve 25 g sodium sulfite in the washed solution.
4. Store the solution in a dark bottle (maximum storage time, 1–2 wk).

Sulfur clean-up

1. Add 2.0 ml TBA sulfite solution and 2.0 ml isopropanol to the extract, mix for 1 min on a vortex. Sulfur clean-up is complete if precipitation is visible. Add an extra 100 mg sodium sulfite if no precipitation is present and mix 1 min on a vortex. Repeat the addition if necessary.
2. Add 5 ml of HPLC-grade water, mix for 1 min on a vortex.
3. Let the layers separate during approximately 5 min, transfer the hexane layer to a clean collection vial.

4. Add 1 ml hexane to the extract and mix 1 min on the vortex. Let the layers separate and transfer the hexane layer to the clean collection vial. Repeat this step. Evaporate the hexane until approximately 1 ml is left.

Acid silica clean-up

1. Prepare a solution of hexane/diethylether (97/3; v/v).
2. Place a small piece of glass wool in a separation. As the performance of the following steps is column-dependent (see fig. 23.1 for column layout).
3. Fill the column with 5 g of 33% silica and tremble the cells with the engraver pen. Add 5 g of 20% silica and tremble the column once more. Add a small amount of dried sodium sulfate to the top of the column.
4. Elute the column with 20 ml hexane/diethylether solution.
5. Bring the extract on the column as soon as the meniscus reaches the sodium sulfate. Wash the collection vial of the extract twice with approximately 1 ml hexane/diethylether solution.
6. Place a clean collection vial under the column and elute the column with 38 ml hexane/diethylether.
7. Evaporate the hexane until less than 1 ml is left.
8. Proceed to solvent exchange.

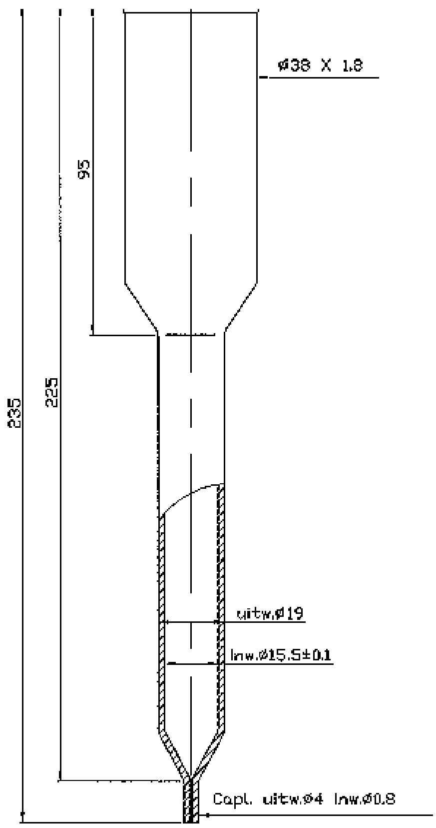


Figure 23.1. Layout of borosilica column for use with acid silica clean-up.

23.3.2.2 ER-CALUX

This section describes the clean-up of deconjugated fish bile extract for use in the ER-CALUX assay. Steps are numbered B.5, B.6, etc, referring to the fish bile extraction procedure above.

B.5) Add 100 µl 1N HCl to each glass test tube containing the deconjugated bile sample (see B.1 above). Stir well (vortex).

B.6) Add 2 ml ethyl acetate to each test tube. Vortex for 1 min, followed by centrifugation for 5 min at 3800 rpm.

B.7) Remove the ethyl acetate fraction using a Pasteur pipette and transfer this to a new test tube. If protein formation is observed between the water and solvent phases, precipitate this protein by adding 500 µl of isopropanol after centrifugation.

B.8) Repeat steps B.6 and B.7 three times, with exception of the isopropanol step.

B.9) Concentrate the collected ethyl acetate fractions and evaporate to a small drop under a gentle N_2 gas flow at 37°C.

B.10) Transfer the concentrated extract to a conical glass vial.

B.11) Rinse the glass test tube three times with ethyl acetate, and transfer the rinses to the conical vial.

B.12) Evaporated the ethyl acetate to dryness at 37°C under a gentle stream of nitrogen.

B.13) Proceed to solvent exchange.

23.3.3 Solvent exchange

Bakker *et al*. (2007) developed criteria and evaluated cosolvents for bioassays. The ideal cosolvent or carrier solvent used for ecotoxicity testing should meet the following criteria: (i) effective: sufficiently high solubility of target compounds, (ii) water-miscible: the carrier solvent must be water-miscible, and (iii) non-toxic: the carrier solvent should have little or no adverse effects on test organisms or cells at typical test concentrations in aqueous media (usually 0.1% v/v). The authors tested ten different solvents, with the following final ranking for the first five solvents:

SOLVENT	FINAL RANK
Dimethylsulfoxide (DMSO)	1
2-Propanol	2
Acetone	2
Methanol	4
Ethanol	5

The following general solvent-exchange protocol is applicable to all five solvents:

- Transfer the remaining cleaned extract to a conical vial and evaporate until a small meniscus of it is left (approximately 20 µl).
- Wash the collection vial twice with at least 0.5 ml DCM or other appropriate solvent, and transfer this to the conical vial (evaporate between washes; do not let the vial fall dry).
- Evaporate the extract until the meniscus reaches the bottom of the conical vial and then add 50 µl of cosolvent.

23.4 Preparation of extract test dilutions for *in vivo* bioassay

The following procedure should be employed when using the prepared extract(s) for standard *in vivo* bioassay testing. This approach is focused on microscale tests with a typical test volume of no more than 5 ml.

Once prepared using the above extraction procedure, the extract must be stored at –20°C until bioassayed, and should not be stored for longer than 12 wk.

A stock solution is made with the concentrated extract using the appropriate dilution water (i.e. aerated seawater or freshwater), from which an appropriate series of concentrations will be prepared. The preparation of the stock solution is important: typically 5 ml of extract in solvent is concentrated by evaporation to 20 µl. The concentration series must be made up on the day of testing, and the ratio between the concentrations should not exceed 2.2 (usually log).

The stock solution must be shaken vigorously, stirred on a magnetic stirrer for at least 30 min, or placed in the ultrasonic bath for 10 min to ensure that all of the chemical/compound(s) within the extract are in solution. The solvent concentration in the final test solution must not exceed 0.1 ml l^{-1}, with all test concentrations containing the same amount of solvent. A solvent control of the appropriate solvent at the same concentration must be used. All controls and test concentrations must have at least three replicates. The salinity, pH, temperature, and dissolved oxygen concentration of the test concentrations must be checked prior to testing and corrected to within the specific parameters of the bioassay as appropriate.

Where possible, the concentrations selected should cover a range from low concentrations with no effect on the test organism relative to the control, intermediate effects, and complete 100% effect. Clearly, this may require an initial sighting test prior to conducing a definitive test. This will allow the calculation of the NOEC, LOEC and EC_{50} values with greater precision.

23.4.1 Preparation of extracts for cell lines

DMSO is the recommended solvent for use with cell line exposures. The concentration of solvent in the final test volume should not exceed 1% (v/v).

23.4.2 Confounding factors

For small test volumes, evaporation of the test solution can be a problem as the volume-to-air-surface ratio is high, and particularly if the test temperature is high (e.g. >15°C). Precautions should be taken to avoid evaporation and also the contaminant crossover that can occur in multiwell plates. In this respect, a short exposure time is desirable: Test duration is typically not greater than 48 h, although there are some exceptions, such as bioassays with algae, which may need a 72 h exposure.

The surface-area-to-volume ratio of the test container is high, and some contaminants may preferentially adhere to surfaces such as polystyrene. For this reason, glass test containers should be used in preference to plastic.

23.5 Conclusions

Whatever the matrix, extraction procedures generally produce small volumes and, therefore, small-scale bioassay procedures are required for testing. In most cases, the recommended procedures are adapted from well-established protocols. The choice of test species will depend on the purpose of the study and the availability of test organism.

Bioassays frequently used for testing extracts are listed in Table 23.2.

Table 23.2. Bioassays used for extract testing

	Test organism	Test volume	Number of organisms/cells per test vessel	Reference
In vivo	Mussel embryo	1–5 ml	50 per ml	ASTM724
	Oyster embryo	1–5 ml	50 per ml	ASTM724
	Sea urchin	1–5 ml	40 per ml	ASTM1563
	Microalgae (freshwater and seawater)	1–5 ml	5×10^6 cells l^{-1}	ISO8692, ISO10253
	Macrophyte germination	1–5 ml	500–1 000 zygotes ml^{-1}	Brooks *et al.* (2008)
	Daphnia	1–5 ml	1 per test vessel	ISO6341
	Acartia/ Nitocra	5 ml	5 per test vessel	ISO14669
	Tisbe	5 ml	5 per test vessel	ISO14669
	Fish embryo	2–5 ml	1 per 2-ml test vessel	OECD draft guideline
In vitro	YES, YAS, anti-YES, anti-YAS	200 µl	0.8×10^6 cells ml^{-1}	Tollefsen *et al.* (2007)
	ER-CALUX	200 µl	$5–10 \times 10^5$ cells ml^{-1}	Legler *et al.* (2003)
	Primary cell cultures	200 µl	5×10^5 cells ml^{-1}	Tollefsen *et al.* (2003)
	Cell lines	200 µl	$5–10 \times 10^5$ cells ml^{-1}	

Matrix	Procedure	Bioassay	Reference
Sediment	ASE, DCM, acetone	ER-CALUX	Houtman *et al.* (2007)

In all of the above test methods, appropriate reference materials should be tested as stated in the specific test protocols.

24 Background document: *in vitro* DR-Luc/DR-CALUX bioassay for screening of dioxin-like compounds in marine and estuarine sediments

Dick Vethaak and Ian M. Davies

24.1 Executive summary

Applicability across the OSPAR maritime area. The *in vitro* DR-Luc assay (also called DR-CALUX, a trademark of BDS (Amsterdam, The Netherlands), hereafter generally referred to as DR-Luc), is a rapid, extremely sensitive, and cost-effective tool for screening marine and estuarine sediments for dioxin-like compounds, including congeners of polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and chlorinated biphenyls (PCBs). The DR-Luc assay is available for immediate deployment within the OSPAR Joint Assessment and Monitoring Programme (JAMP) Coordinated Environmental Monitoring Programme (CEMP). The DR-Luc assay has been recommended by ICES and is of sufficient standing, in terms of methodological development and application, for uptake across the whole OSPAR area.

Quality assurance. QA procedures are in place and interlaboratory performance studies are organized frequently, but there remains a need for QA within international programmes, such as BEQUALM. The methodology for DR-Luc and related extraction protocols are well developed and available through *ICES TIMES* series documents. DR-Luc data can be submitted to the ICES database for subsequent assessment, as appropriate, by ICES/OSPAR.

Influence of environmental variables. In general, there is little influence of environmental variables on the test conditions and bioassay response; the use of extracts will reduce any disturbing factors. Sediments should be sampled according to guidelines for chemical analysis to take account of organic carbon (OC) content and particle size.

Thresholds and assessment tools. Three assessment classes were derived for DR-Luc based on silica clean-up per 24-h exposure: (i) a background response <10 pg TEQ (toxic equivalent quotient) g^{-1} dry wt, (ii) an elevated response (warning level) of >10–<40 pg TEQ g^{-1} dry wt, and (iii) a high and cause-for-concern response of >40 pg TEQ g^{-1} dry wt.

Synergism between CEMP/Marine Strategy Framework Directive (MSFD) and Water Framework Directive (WFD). The DR-Luc bioassay can be immediately applied in offshore and coastal sediments and is equally suitable for estuarine and freshwater sediments. As such, the use of DR-Luc can play a role in linking the MSFD with the WFD.

24.2 Background

Dioxin levels in the marine environment have declined significantly in the past two decades as a result of reductions in emissions from man-made sources (Rappe, 1996; Aylward and Hays, 2002). However, degradation in the environment is slow and, therefore, dioxin-like compounds from past releases are expected to remain in the environment for many decades. The term "dioxin-like compounds" refers to a group of structurally similar congeners known as polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), and some polychlorinated biphenyls (PCBs; see also OSPAR Background Document on dioxins; OSPAR, 2007).

Dioxin-like compounds are unintentionally released by-products of the combustion of chlorinated compounds in the environment. In addition, there are a number of other compounds that exhibit dioxin-like properties, such as polybrominated biphenyls (PBBs) and polycyclic aromatic hydrocarbons (PAHs).

In the past two decades, there has been growing environmental concern regarding dioxins and other compounds that have dioxin-like properties. The major concerns with dioxin-like compounds are their effects on wildlife and human health owing to their resistence to degradation and ability to be bioaccumulated (van den Berg *et al.*, 1998). They have also been shown to produce a wide variety of toxic and biochemical effects via aryl hydrocarbon (Ah) receptor-mediated signalling pathways (Mandal, 2005). The effects on laboratory animals and wildlife include developmental and reproductive effects, immunotoxicity, neurotoxicity, and carcinogenesis (for more details and references, see OSPAR, 2007). Animals at particular risk are fish-eating top predators, such as otters (Murk *et al.*, 1998), seals (Vos *et al.*, 2000), and birds (Bosveld *et al.*, 1995; Henshel, 1998). The effects of dioxin-like compounds in humans include high acute toxicity, skin lesions, developmental and reproductive abnormalities, and probably cancer (WHO, 2000; Aylward *et al.*, 2003; Heilier *et al.*, 2005). It has been shown that aquatic organisms can ingest dioxin-like compounds that have been flushed into surface water from land, providing a potential pathway into the food chain (Leonards *et al.*, 2008).

Dioxin-like compounds share (at least initially) a common mode of action by binding to the Ah receptor, which mediates and interacts with a series of biological processes, including cell division and growth and homeostatic functions (Puga *et al.*, 2005; Stevens *et al.*, 2009). Of 75 PCDD congeners, only seven have been identified as having dioxin-like toxicity (Liem and Zorge, 1995) and only 10 of the 135 PCDFs are thought to have dioxin-like toxicity (Aarts and Palmer, 2002). For PCBs, only 12 of the 209 congeners are thought to have dioxin-like toxicity (Liem and Zorge, 1995). The Ah receptor or dioxin receptor-based *in vitro* assay DR-Luc (also known as DR-CALUX (Dioxin Response Chemical-Activated LUciferase gene eXpression, a trademark of BDS, Amsterdam, The Netherlands) is considered to be the most useful *in vitro* bioassay technique for screening for dioxin-like compounds. However, the induction of CYP1A/EROD in fish liver (see OSPAR background document on CYP1A/EROD activity) and chronic *in vivo* bioassays (Foekema *et al.*, 2008) may also be relevant. An advantage of the application of these *in vitro* bioassays (using extracts), as compared with CYP1A/EROD, is that they are independent of species differences and environmental influences, and so are applicable in a generic way. The use of extracts will minimize the influence of environmental variables and reduce any disturbing factors. Sediments should be sampled according to guidelines for chemical analysis to take account of OC content and particle size.

24.3 DR-Luc as bioassay for dioxin-like compounds

The DR-Luc is a reporter-gene assay that was developed by Wageningen University (Aarts *et al.*, 1995; Murk *et al.*, 1996) and is distributed as DR-CALUX by Bio Detection System (BDS, Amsterdam, The Netherlands). This system incorporates a reporter-firefly gene into a cultured rat H4IIE hepatoma cell line. Exposed to dioxin-like compounds, this system produces the enzyme luciferase, which reacts with luciferin and emits light of a characteristic wavelength with intensity proportional to the dioxin concentration. The mode of action of Ah receptor-mediated action is illustrated and further explained in Figure 24.1.

The DR-Luc is a highly sensitive reporter-gene assay, allowing detection of 1 pM TCDD (Murk *et al*., 1996). As such, the DR-Luc assay for dioxin-like substances is much cheaper and faster than the conventional chemical HRGC-MS4 methods.

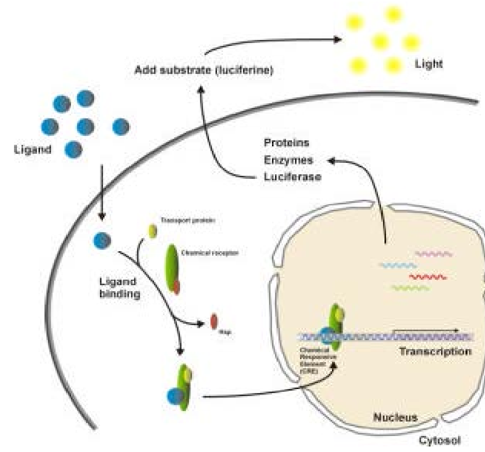


Figure 24.1. Activation of the Ah receptor-mediated luciferase gene in the DR-Luc bioassay (figure by RIKZ, 2006). Following activation of the receptor, the ligand–Ah receptor complex translocates to the nucleus of the cell, where it binds to specific DNA sequence, the so-called DREs. The binding of the ligand–Ah receptor complex to the DREs results in changes in the expression of DR-Luc associated genes (e.g. cytochrome P4501 A1). These changes in gene expression result in the disturbance of normal cell physiology. Following exposure of the cells to dioxin or dioxin-like compounds, the enzyme luciferase is produced. Addition of the substrate luciferin to lysed cells results in light production. The amount of light produced is recorded in a luminometer and is interpolated on the amount of 2,3,7,8-TCDD toxic equivalents standard curve to which the genetically modified H4IIE cells were exposed.

The response of DR-Luc is a measure of toxic potency and is usually expressed as TEQs relative to the biological response in the DR-Luc bioassay of the most toxic compound 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD). The TEQ values are calculated on the basis of concentrations of individual congeners, as determined by HRGC-MS (see OSPAR, 2007).

24.4 Applicability of *in vitro* DR-Luc bioassay across the OSPAR maritime area

The DR-Luc assay is a suitable screening method for dioxins and dioxin-like-PCBs in feed and food [for example, a survey in The Netherlands to control the dioxin levels in eel (Hoogenboom *et al*., 2006)], risk assessment and management of saline and freshwater whole effluents (e.g. Oris and Klaine, 2000; Power, 2004), and for dredged material (Stronkhorst *et al*., 2002, 2003; Schipper *et al*., 2010).

The DR-Luc assay is widely recognized within Europe as an efficient way to assess sediment quality (e.g. Stronkhorst *et al*., 2003; Houtman *et al*., 2004, 2006; Hurst *et al*., 2004; Legler *et al*., 2006a,b; van den Brink and Kater, 2006; Sanctorum *et al*., 2007; Schipper *et al*., 2009, 2010; Hamers *et al*., 2010). Bioassays are also applied on the national level by several countries (ICES, 2010b). Findings from several studies demonstrate this bioassay to be of value in both inshore and offshore regions, for example, a high DR-CALUX response was found in surface sediments at the Oyster Grounds, (an offshore region in the southwestern North Sea) that could be linked with the occurrence of larger PAHs (4–6 rings; Klamer *et al*., 2005b).

From the above studies, it was concluded that the method could be useful as a screening method associated with a specific action level, because if the bioassay results are below the action level, it is most likely that results by the chemical method would also have been below. Good correlations were usually observed between DR-Luc/CALUX bioassay results obtained on marine biological matrices and results obtained from the use of advanced chemical methods (Windal *et al.*, 2002; Hoogenboom, 2002). An intra- and interlaboratory study using CALUX for analysis of dioxins and dioxin-like chemicals in dredged sediments also concluded that the tool was accurate and reliable for monitoring coastal sediments (Besselink *et al.*, 2004).

The uptake of other *in vitro* reporter-gene bioassays that can be applied together with DR-Luc in a test battery, such as *in vitro* bioassays for endocrine disruption (ER-Luc, YES, YAS) and for immunotoxic and neurotoxic compounds (Hamers *et al.*, 2010), as well as general toxicity (e.g. Microtox SPT assay), should also be encouraged.

24.5 Introduction of DR-Luc bioassays to the CEMP and status of quality assurance

The DR-Luc assay is proposed in the OSPAR JAMP Guidelines as a suitable specific biological effect method for monitoring PCBs, polychlorinated dibenzodioxins, and furans, and also as a suitable method for general biological effect monitoring. In addition, the DR-Luc assay can be used in toxicity reduction evaluation (TRE), toxicity identification evaluation (TIE), and effects-directed analysis (EDA) procedures (Burgess, 2000) as well as sediment toxicity profiling (Hamers *et al.*, 2010).

A number of papers have been published describing the validation of the DR-Luc bioassay and describing the correlation between DR-Luc and HRGC-MS-derived 2,3,7,8-TCDD TEQs (van den Berg *et al.*, 1998; Stronkhorst *et al.*, 2002; Besselink *et al.*, 2003; van Loco *et al.*, 2004). It has been shown that frequent participation in interlaboratory exercises improves performance (de Boer and Wells, 1996; Besselink *et al.*, 2004), but there remains a need for QA to be established as routine within international programmes such as BEQUALM.

The protocol for the DR-Luc assay, including methods for sediment extraction, is available in the *ICES Techniques in Marine Environmental Sciences* series on biological effects of contaminants.

24.6 Synergism between CEMP, MSFD, and WFD

Although *in vitro* DR-Luc and other bioassays are not included as ecological quality elements in the monitoring for the Water Framework Directive (WFD; WFD CIS, 2003), it is generally accepted that they will be able to contribute to investigative monitoring and the pressures and impacts/risk assessment process (this is especially true for chronic water and sediment bioassays). Further chemical analysis can be combined with water bioassays at smaller interval time-points for the purposes of trend monitoring. In this way, bioassays can be used as a partial replacement for chemical analysis of priority and/or other relevant substances and prioritizing locations for further chemical analysis. This "bioanalysis approach" can lead to more cost-efficient and cost-effective monitoring and would put the precautionary principle called for in the WFD into practice. Pilot studies carried out in The Netherlands to explore these possibilities have had promising results (e.g. Maas and van den Heuvel-Greve, 2005). It can be concluded that clear opportunities exist for synergism between the CEMP or the MSFD and WFD for the application of DR-Luc bioassay in coastal and estuarine areas. In addition to being a cost-effective technique, the DR-Luc will strengthen the monitoring capacity for dioxin-like compounds and better understand the status of dioxin pollution in marine environment.

24.7 Thresholds and assessment tools

Three assessment classes were derived for DR-Luc based on silica clean-up per 24-h exposure: (i) a background response <10 pg TEQ g^{-1} dry wt, (ii) an elevated response (warning level) of >10–<40 pg TEQ g^{-1} dry wt, and (iii) a high and cause-for-concern response of >40 pg TEQ g^{-1} dry wt. These AC are based on datasets and experience from the UK, Belgium, and the Netherlands. It is advised that these AC should be further refined as more data become available.

24.8 Derivation of assessment criteria for DR-Luc

The most conservative criteria for dioxin-contaminated sediments are from Canada (4 pg TEQ g^{-1})(AEA Technology, 1999) and from the US (2.5 pg TEQ g^{-1})(Thain *et al.*, 2006; Table 24.1). These criteria are "screening levels" which, if exceeded, trigger further investigation at a particular site. Exceeding a screening level does not immediately imply a health risk. Any risk will be relative to the exposure assumed in the derivation of the guideline and the exposure likely in the actual situation. In some international guidelines concerning the regulation of dioxins, sediments are divided pragmatically into "clean" and polluted locations on the basis of existing measurements of *in vitro* bioassays, as with the DR-Luc/DR-CALUX (Stronkhorst *et al.*, 2002). The expected serious chronic effect levels are the average maxima found at locations assumed to be "clean". For example, DR-CALUX measurements showed in Dutch surface sediments (Stronkhorst *et al.*, 2002; Klamer *et al.*, 2005b) from major Dutch "clean" offshore sites up to 70 miles offshore, with values at three offshore sites below 10 pg g^{-1} (6.9 and 8, respectively). Based on this, a background response level has been derived of <10 pg TEQ g^{-1} dry wt. In the analysis of dioxins and dioxin-like chemicals in sediments, ranges of TEQs in dredged sediments from rivers in the coastal zone were 12–70 pg TEQ g^{-1} dry wt and, on average, 24 pg TEQ g^{-1} dry wt (Schipper *et al.*, 2010). In several studies from the Dutch and Belgium coastal zone, a range of TEQ values was observed between 9 and 27 pg TEQ g^{-1} dry wt (Klamer *et al.*, 2005b) and 10–42 pg TEQ g^{-1} dry wt sediment (Sanctorum *et al.*, 2007). The level of serious concern is then the average maximum found at locations assumed to be "clean": >40 pg TEQ g^{-1} dry wt. The elevated response has been derived as a warning level of >10–<40 pg TEQ g^{-1} dry wt.

Table 24.1. International dioxin guidelines (TCDD TEQ) in sediments (dry weight basis)

Country	Maximum allowable concentration (dry weight basis)	Comments	Reference
Vietnam	150 pg g^{-1} TEQ	Dioxin heavily contaminated sites (sediments)	Hatfield Consultants (2009)
USA	2.5 pg g^{-1} TEQ	Protection level	Thain *et al.* (2006)
Canada	4 pg g^{-1} TEQ	Protection of ecological receptors	AEA Technology (1999)
Germany	5 –10 pg g^{-1} TEQ	Protection of human receptors	AEA Technology (1999)
The Netherlands	50 pg g^{-1} TEQ	Target value	Stronkhorst *et al.* (2002)

24.9 Conclusions

DR-Luc/DR-CALUX *in vitro* bioassays for dioxin-like compounds are available for immediate deployment within the OSPAR JAMP CEMP. These bioassays have been recommended by ICES and are of sufficient standing in terms of methodological development, ease of use, and application for uptake across the whole OSPAR area. QA procedures are in place, and continuation of QA should be by BEQUALM. Therefore, bioassay data can be submitted to the ICES database for subsequent assessment, as appropriate, by ICES/OSPAR.

The range of *in vitro* bioassays needs to be expanded to include oestrogenic and androgenic compounds, as well as neurotoxic and immunotoxic compounds and cell-based general toxicity assays.

Appropriate protocols for DR-Luc and associated extraction methods are available through the *ICES TIMES* series.

Assessment criteria for the DR-Luc bioassay are available.

It is recommended that OSPAR lists the DR-Luc/DR-CALUX bioassay as a Category-II-rated method in the JAMP CEMP programme and integrated monitoring scheme.

25 Assessment criteria for imposex in marine gastropods affected by exposure to organotin compounds

Matt Gubbins, Jakob Strand, John Thain, and Ian M. Davies

An OSPAR technical annex to the Joint Assessment and Monitoring programme (JAMP) already provides background and guidelines to the measurement of imposex and intersex in marine gastropods affected by exposure to organotin compounds (primarily tributyltin, TBT; OSPAR, 2002). Assessment criteria have also already been defined for the effects of organotins in gastropod molluscs (OSPAR, 2004). A six-class scheme (A–F) was devised based on the levels of vas deferens sequence index (VDSI) in populations of dogwhelks (*Nucella lapillus*) that related to relevant concentrations of TBT (close to zero and at EAC) and effects (reduced growth, recruitment, sterility, and death) in the most sensitive taxa (Table 25.1).

Table 25.1. Relevance of assessment classes relating to *Nucella lapillus*, the most TBT-sensitive species for which guidelines and assessment criteria are available

Assessment class	Nucella VDSI	Effects and impacts
A <BAC	VDSI = <0.3	The level of imposex in the more sensitive gastropod species is close to zero (0—~30% of females have imposex) indicating exposure to TBT concentrations close to zero, which is the objective in the OSPAR Hazardous Substances Strategy
B >BAC <EAC	VDSI = 0.3—<2.0	The level of imposex in the more sensitive gastropod species (~30—~100% of the females have imposex) indicates exposure to TBT concentrations below the ecotoxicological assessment criteria derived by OSPAR for TBT. For example, adverse effects in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT are predicted to be unlikely to occur.
C >EAC	VDSI = 2.0— <4.0	The level of imposex in the more sensitive gastropod species indicates exposure to TBT concentrations higher than the EAC derived for TBT. For example, there is a risk of adverse effects, such as reduced growth and recruitment, in the more sensitive taxa of the ecosystem caused by long-term exposure to TBT
D >EAC	VDSI = 4.0–5.0	The reproductive capacity in the populations of the more sensitive gastropod species, such as *Nucella lapillus*, is affected as a result of the presence of sterile females, but some reproductively capable females remain. For example, there is evidence of adverse effects that can be directly associated with the exposure to TBT
E >EAC	VDSI = >5.0	Populations of the more sensitive gastropod species, such as *Nucella lapillus*, are unable to reproduce. The majority of, if not all, females within the population have been sterilized
F >EAC	VDSI = –	The populations of the more sensitive gastropod species, such as *Nucella lapillus* and *Ocinebrina aciculata*, are absent/expired

Adapted from OSPAR (2004) to show equivalence of OSPAR assessment classes to background assessment criteria (BAC) and environmental assessment criteria (EAC) thresholds for application to the ICES/OSPAR integrated assessment framework.

By using data on imposex/intersex in sympatric populations of affected gastropods of different species, the assessment criteria were able to be extended to a range of species used for environmental monitoring across the ICES area and set for VDSI in *Nassarius reticulatus*, *Buccinum undatum*, and *Neptunea antique*, and intersex sequence index (a measure of intersex, not imposex) in *Littorina littorea* (Table 25.2).

However, some further guidance is required to allow application of these assessment classes to the ICES/OSPAR integrated assessment framework. Background assessment criteria (BAC) and environmental assessment criteria (EAC) levels must be set to allow compatibility with the rest of the integrated approach.

It is proposed here that a BAC of VDSI = 0.3 be used for *Nucella lapillus* and *Neptunea antiqua* only. At levels below this, it is difficult to determine effects above exposure to background (zero) concentrations of organotins. Effects measurements in the other species are not sufficiently sensitive to be used to determine samples where the BAC are exceeded.

EAC should be set as VDSI = 2 in *Nucella lapillus* and *Neptunea antique*, but at VDSI = 0.3 in *Buccinum undatum* and *Nassarius reticulatus* (the boundary between OSPAR classes B and C). These values represent an effect equivalent to exposure to the same concentrations of TBT across all four species and represent the expected effects from exposure of the most sensitive species to TBT at the EAC concentration. At these levels, other effects of TBT are expected on sensitive taxon, for example, on growth and recruitment. These levels of effect also match the OSPAR ecological quality objective (ECoQO) for imposex in *Nucella lapillus*. Intersex in *Littorina littorea* is considered too insensitive for application of BAC and EAC.

Table 25.2. OSPAR biological effect assessment criteria for TBT. Assessment criteria for imposex in *Nucella lapillus* are presented alongside equivalent VDSI/ISI values for sympatric populations of other relevant species

Assessment class	*Nucella*	*Nassarius*	*Buccinum*	*Neptunea*	*Littorina*
	VDSI	VDSI	VDSI	VDSI	ISI
A (<BAC)	<0.3	<0.3	<0.3	<0.3	<0.3
B (>BAC <EAC)	0.3–<2.0			0.3–<2.0	
C (>EAC)	2.0 <4.0	0.3–2.0	0.3–2.0	2.0–4.0	
D (>EAC)	4.0–5.0	2.0–3.5	2.0–3.5	4.0^	0.3– <0.5
E (>EAC)	>5.0	>3.5	>3.5		0.5–1.2
F (>EAC)					>1.2

Adapted from OSPAR (2004) to show equivalence of OSPAR assessment classes to BAC and EAC thresholds for application to the ICES/OSPAR integrated assessment framework.

VDSI, vas deferens sequence index; ISI, intersex sequence index.

Potentially, other gastropod species could be included in these assessment criteria for TBT effects if interspecies correlations for sympatric populations have been determined. This can be useful for a further expansion of a harmonized assessment level for TBT effects into other sea areas (e.g. the mudsnail *Hydrobia ulvae* could be used in the Baltic Sea, where it is the only gastropod species known that can be used for TBT-specific effects studies across different subregions).

26 Technical annex: sampling and analysis for integrated chemical and biological effects monitoring in fish and shellfish

Dick Vethaak, Thomas Lang, and Ian M. Davies

26.1 Introduction

ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON) and associated groups have progressively developed an integrated approach to the use of biological effects and chemical measurements in environmental monitoring and assessment to meet the objectives of the OSPAR Hazardous Substances Strategy. In relation to hazardous substances, the OSPAR Joint Assessment and Monitoring Programme seeks to address the following questions:

- What are the concentrations in the marine environment, and the effects, of the substances on the OSPAR List of Chemicals for Priority Action ("priority chemicals")? Are they at, or approaching, background levels for naturally occurring substances and close to zero for man-made substances?
- Are there any problems emerging related to the presence of hazardous substances in the marine environment? In particular, are any unintended/unacceptable biological responses, or unintended/ unacceptable levels of such responses, being caused by exposure to hazardous substances?

The primary means of addressing these questions on an OSPAR-wide basis is the CEMP (OSPAR Agreement 2005, 5). Advice on updated Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects were presented by ICES to OSPAR in 2011 in response OSPAR request 2008/8.

The integrated approach described in the Guidelines is based on recommendations of sets of measurements that could be used to investigate the effects of contaminants on sediment, fish, or shellfish (mussels, gastropods), and overviews of these are included in the Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects. These reflect the wide experience of the monitoring of the concentrations of priority contaminants in sediment and biota, and the benefits of combining this with the developing experience of the use of biological effects measurements in monitoring programmes. More detailed schemes for integrated monitoring are included in the Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects, and are shown in Figures 26.1 and 26.2.

As indicated in the Guidelines, the contribution made by an integrated programme, involving both chemical and biological effects measurements, is primarily that the combination of the different measurements increases the interpretive value of the individual measurements. For example, biological effects measurements will assist in the assessment of the significance of measured concentrations of contaminants in biota or sediments. When biological effects measurements are carried out in combination with chemical measurements (or additional effects measurements), this will provide an improved assessment as a result of the possible identification of the substances contributing to the observed effects.

The structure of each of the schemes recognizes that a full integrated assessment requires the integration of a variety of chemical measurements (concentrations of contaminants in the fish or mussels) and biological effects data.

It is well recognized that some particular contaminants or groups of contaminants can have characteristic biological effects. The classic example of a highly specific response to a contaminant is that of the effects of tributyltin (TBT) compounds in inducing imposex or intersex in gastropod mollusc species. These responses have been widely used as an assessment of the environmental significance of tributyltin compounds, and are the topic of an OSPAR EcoQO. Although it is theoretically possible for other substances to disrupt the hormonal systems of snails in a similar way, it is generally accepted that TBT is the primary marine contaminant responsible for the effects.

There is clearly great attraction in the recognition of a highly specific response to a particular narrow class of contaminants, especially if chemical analysis at concentrations known to be associated with the effects is difficult. However, such close relationships are generally rare. For example, a range of effects measurements have been applied to the effects of planar organic contaminants in the sea:

- the concentration of polycyclic aromatic hydrocarbon (PAH) metabolites in fish bile;
- cytochrome P450 1A (CYP1A)/EROD induction;
- indices of genotoxicity (e.g. DNA adducts of PAH, comet assay, micronucleus assay);
- liver (microscopic) neoplasms; and
- liver histopathology.

However, these effects show varying degrees of specificity for PAH as opposed to other planar organic contaminants, such as planar chlorinated biphenyls or dioxins. The concentration of PAH metabolites in fish bile is clearly specific to the PAH compounds detected, but CYP1A/EROD induction is a property of a range of groups of compounds.

In general, it is found that, although subcellular responses can commonly be linked to substances that can induce the response, measurements of whole organism, effects are much less contaminant specific. However, they are often more closely linked to the potential to cause effects at population level, through reduction in survival or reproductive capacity. This gradation is reflected in the integrated monitoring frameworks and in Figures 26.1 and 26.2 under the headings of subcellular responses, tissues responses, and whole-organism responses. Subcellular responses, such as EROD, bile metabolite concentrations, and metallothionein, are recognized as biomarkers of exposure to contaminants, whereas whole-organism and tissue-level responses are more clearly markers of effect.

26.2 Sampling and analysis strategies for integrated fish and bivalve monitoring

The integration of contaminant and biological effects monitoring requires a strategy for sampling and analysis that includes:

- sampling and analyses of same tissues and individuals;

- sampling of individuals for effects and chemical analyses from the same population as that used for disease and/or population structure determination at a common time;
- sampling of water, the water column, and sediments at the same time and location as collecting biota; and
- more or less simultaneous sampling for and determination of primary and support parameters (e.g. hydrographic parameters) at any given location.

Examples of sampling strategies for the integrated fish and shellfish schemes are shown in Figures 26.1 and 26.2. The numbers of individual organisms required are driven primarily by the assessment of external diseases and macroscopic liver nodules (fish) and histopathology (bivalves), because these require the largest number of individuals. A subsample of individuals within the primary sample is further sampled for liver histopathology (fish) and biomarkers (fish and bivalves) to meet the first two requirements above.

In the specified target species, further subsampling of the same individuals for chemical analysis is often restricted by insufficient remaining tissue, e.g. liver in fish. In order to meet the second requirement, subsamples for chemical analysis are taken from the same combined hauls/population as those for disease/biomarkers.

In order to integrate sediment, water chemistry, and associated bioassay components with the fish and bivalve schemes, sediment and water samples should be collected at the same time as fish/bivalve samples and from a site or sites that are representative of the defined station/sampling area.

Additional integrated sampling opportunities may arise from trawl/grab contents, for example, gastropods for imposex or benthos, and these should be exploited where possible/practicable.

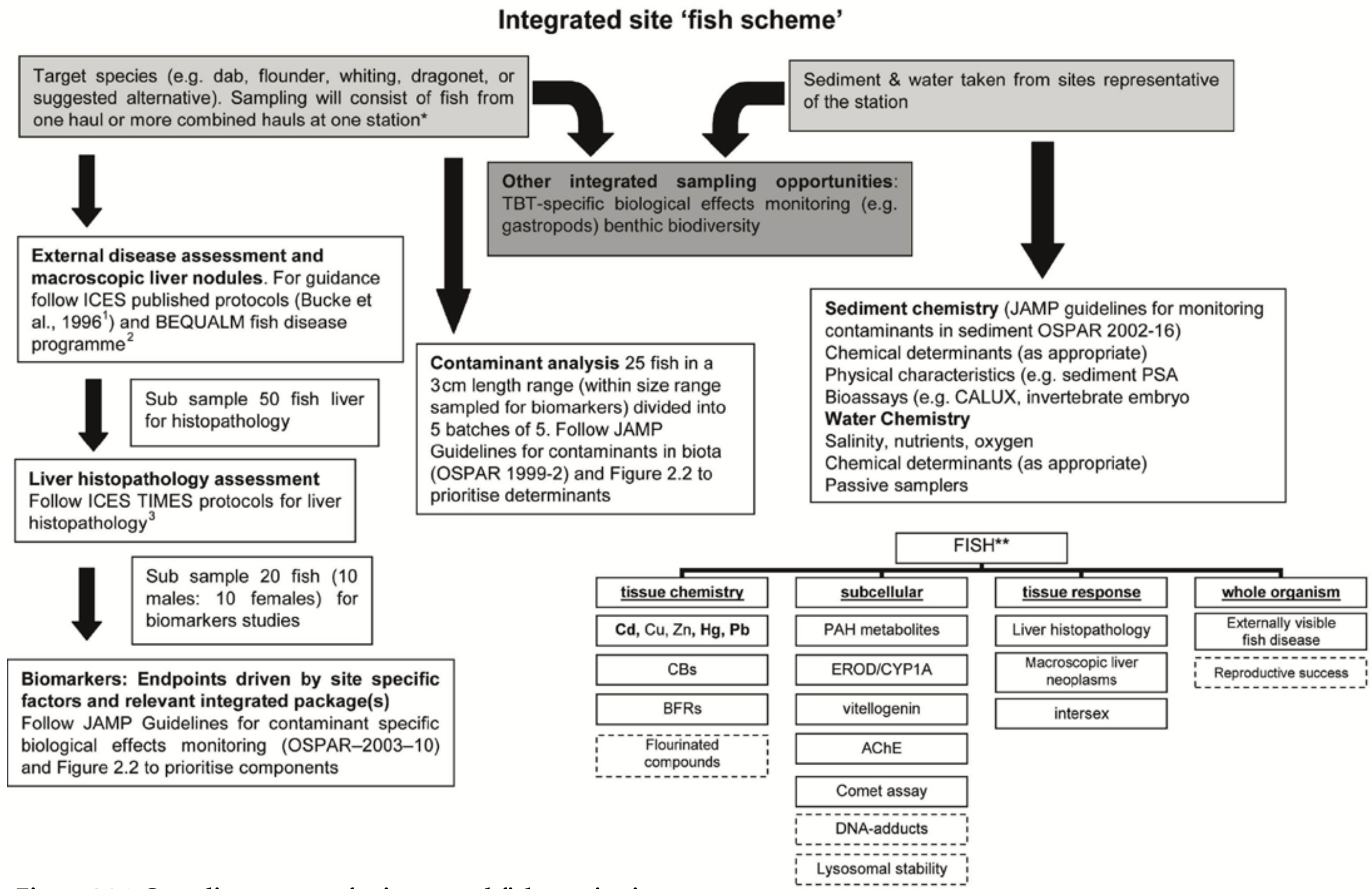


Figure 26.1. Sampling strategy for integrated fish monitoring.

**Figure 2.2 Overview of methods to be included in an integrated programme for selected fish species. (Solid lines – core methods, broken lines – additional methods).

1 Note: A station may be site specific or a larger defined area

2 Bucke, D., Vethaak, D., Lang, T., and Mellergaard, S. 1996. Common diseases and parasites of fish in the North Atlantic: training guide for identification. ICES Techniques in Marine Environmental Sciences, No. 27 pp.

3 BEQUALM: http://www.bequalm.org/fishdisease.htm

4 Feist, S. W., Lang, T., Stentiford, G. D. and Köhler A., 2004. The use of liver pathology of the European flatfish, dab (Limanda limanda L.) and flounder (Platichthys flesus L.) for monitoring biological effects of contaminants. ICES Techniques in Marine Environmental Sciences, No. 38. 47 pp.

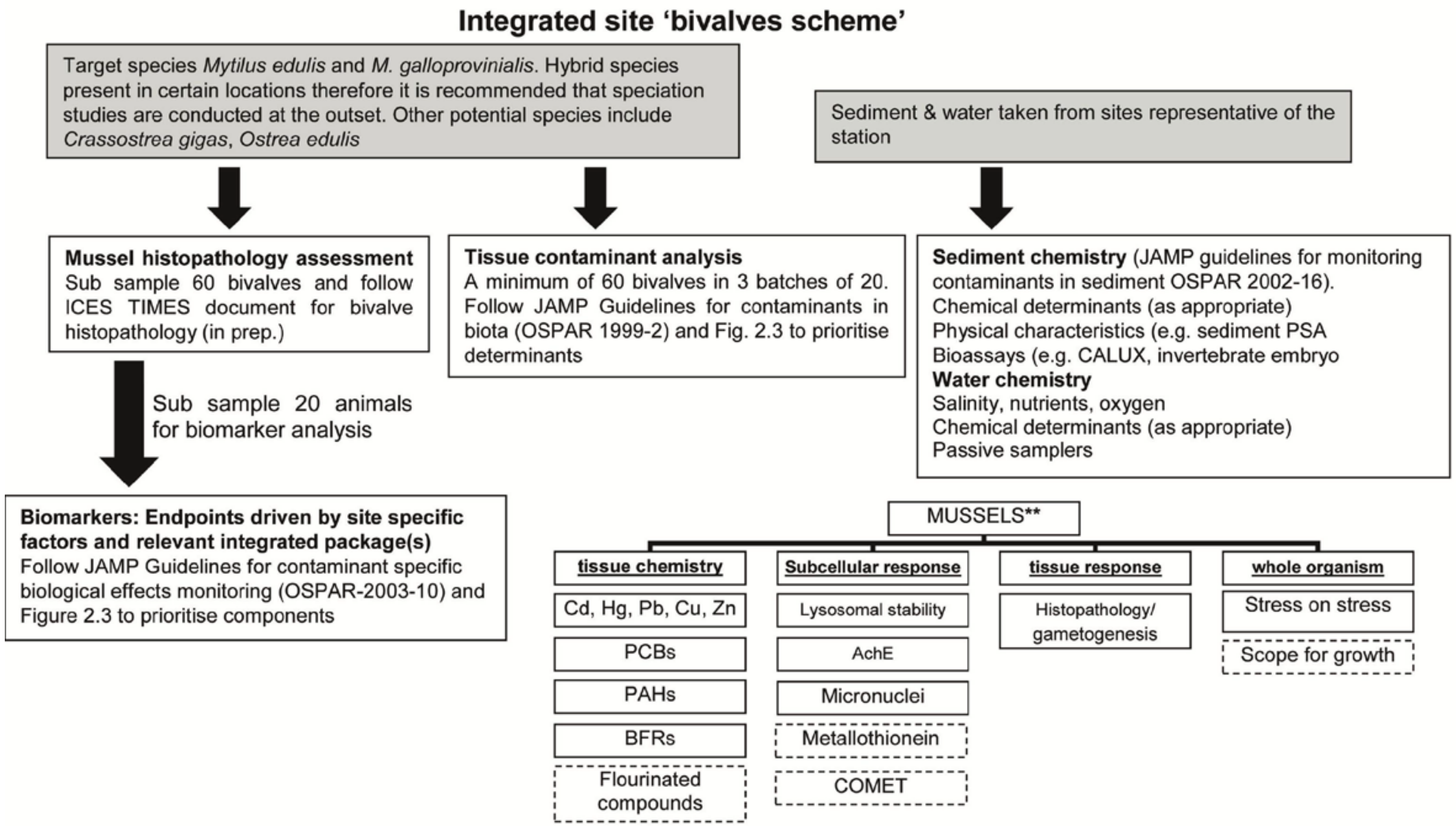


Figure 26.2. Sampling strategy for integrated bivalve monitoring.

** Figure 2.3 Overview of methods to be included in an integrated programme for selected bivalve species. (Solid lines – core methods, broken lines – additional methods).

Guidance on sampling and analysis for integrated monitoring of biological effects and chemical measurements

Some aspects of the details of fish and shellfish sampling and analysis are covered in the OSPAR JAMP Guidelines. Integration of chemical and biological effects data in coordinated monitoring programmes was not a primary consideration when the components of these guidelines were developed. Some revisions have, therefore, been made to ensure that the information correctly covers the requirements for integrating chemical and biological effects sampling.

The following tables address aspects of technical guidance on sampling design and supporting parameters.

Tables 26.1–26.3 cover methods to be used for integrated fish, bivalve, and gastropod monitoring, Tables 26.4 and 26.5 cover methods for monitoring of water and sediments.

Table 26.1. Overview of selected methods for integrated fish monitoring (2007 WKIMON Report, revised).

SUBJECT	PARAMETER	COMMENT
Species	Primary species: dab, flounder, whiting, eelpout Alternative species: plaice, cod, herring, eelpout, hake, dragonet, or other	Alternative species may be used if primary species are not available
Sex	Females and/or males	For certain biomarkers or chemical measurements, only females or only males are used (see relevant JAMP Guidelines)
Health condition	Specimens free of external visible diseases should be used for chemical and biomarker analysis	Certain biomarkers are affected by disease conditions
Size ranges	Dab: ≥15 cm (according to suggested new JAMP Guidelines for externally visible diseases) Flounder: ≥20 cm (according to suggested new JAMP Guidelines for externally visible diseases) Whiting: ≥15 cm (according to suggested new JAMP Guidelines for externally visible diseases) Dragonet: ≥10 cm (according to suggested new JAMP Guidelines for liver histopathology) Eelpout: Pregnant females 15–30 cm, 50 fish per station	For integrated monitoring encompassing chemistry, histopathology, and biomarkers, the mid-size groups are preferable which are: 20–24 cm (dab), 20–29 cm (flounder), 20–24 cm (whiting), 10–15 cm (dragonet)
Sample size	Depending on the parameter measured, according to JAMP Guidelines	Sample sizes have to fulfil statistical requirements for spatial and/or temporal trend monitoring. Preferably, all measurements should be done in individual fish, and pooling should be avoided (with the possible exception of contaminant measurements)
Sampling time and frequency	Sampling for all parameters should be carried out at the same time, outside the spawning season, and at least once a year in the same time-window	Justification is provided in the OSPAR JAMP Guidelines
Sampling location	Sampling for all parameters should be carried out at the same site	The location, size, and number of sampling sites depend on the purpose of the monitoring. For offshore sampling targeted at fish, it is recommended to use ICES statistical rectangles as sampling sites. A number of repeated samplings (= hauls; replicates) should be carried out in each of these rectangles. For coastal and estuarine waters, sites should be selected based on existing WFD and other

		chemical/biological monitoring sites, taking account of potential hot spot areas or areas at risk. The number of sampling sites should be sufficient to reflect the environmental conditions in the survey area, and meet the purposes of the monitoring programme
Chemical determinands	Metals: Hg, Cd, Pb, Cu, Zn CBs: ICES 7 CBs + CB77, CB81, CB126, CB169 + CB105, CB114, CB123, CB156, CB157, CB167, CB189 Brominated flame retardants: congeners of the penta-mix, octa-mix and deca-mix PBDE formulations; hexabromocyclododecane, tetrabromobisphenol-A Lindane TBT	In addition, *in situ* PAH measurements (e.g. using UV-fluorescence spectrometry) may be employed under specific circumstances (e.g. after oil spill or PAH-related point source discharges). In addition to the contaminants already covered by the OSPAR CEMP, there are a number of other compounds from the OSPAR list of Chemicals for Priority Action that should be monitored because of their toxicity and environmental relevance. The list provided is, therefore, not complete
Biological effects measurements	Biological effect techniques as specified in the OSPAR Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects, as in Figures 26.1 and 26.2	Additional opportunities for the inclusion of new methods are likely to emerge through the implementation of MSFD and as science develops.Potential examples include indicators of immunocompetence and embryo malformation
Supporting parameters	Length, weight, gender, age, somatic indices, stage of gonadal maturation, grossly visible anomalies, lesions, parasites, hydrography (temperature, salinity, oxygen content)	In the list, parameters are provided that are known to affect both the biological effects responses and the concentration of contaminants. The data can be of assistance in data interpretation
Haul duration	Haul durations should be harmonized between monitoring authorities. An appropriate value would be 30 min, but may be less than this if conditions require	The purpose is to standardize the stress experienced by fish during capture
Duration and conditions of storage of live fish prior to dissection	Fish should be maintained alive in flowing seawater on the sampling vessel for periods not exceeding 8 h	Storage for longer periods or under poor conditions can stress the fish and alter some biomarker responses

Table 26.2. Overview of selected methods for integrated shellfish monitoring (2007 WKIMON Report, revised)

SUBJECT	PARAMETER	COMMENT
Species	Primary species: *Mytilus edulis* Alternative species: *Mytilus galloprovincialis*, *Crassostrea gigas*, *Ostrea edulis*	The first choice shellfish species is not available in all parts of the OSPAR area. In such cases, other species should be selected, such as oysters. For *Mytilus* sp., speciation studies are recommended in order to confirm species identity
Sex	Females and/or males	For certain biomarkers or chemical measurements, only females or only males are used (see relevant JAMP Guidelines)
Size range	Mussel: ≥40 mm, ideally in the range 40–55 mm. Pacific oyster: 9–14 cm	Based on JAMP Guidelines for chemical monitoring
Sample size	Depending on the parameter measured, according to JAMP Guidelines	Sample sizes have to fulfil statistical requirements for spatial and/or temporal trend monitoring. For some parameters, sample size still has to be defined. Preferably, all measurements should be done in individual mussels, and pooling should be avoided (except where recommended, for example, for the measurement of contaminant concentrations)
Sampling time and frequency	Sampling for all parameters should be carried out at the same time, outside the spawning season, and at least once a year in the same time-window	Justification is provided in the OSPAR JAMP Guidelines
Sampling location	Sampling for all parameters should be carried out at the same site	The location, size, and number of sampling sites depend on the purpose of the

		monitoring. For coastal and estuarine waters, sites should be selected based on existing sites used for WFD or other purposes, taking account of hot spot areas and areas at potential risk. The number of sampling sites should be sufficient to reflect the environmental conditions in the survey area, and meet the purposes of the monitoring programme. For coastal and offshore studies, caging of mussels should be considered
Chemical determinands	Metals: Hg, Cd, Pb, Cu PAHs: EPA 16 + NPD CBs: ICES 7 + CB 77, 81, 126, 169 + CB 105, 114, 123, 156, 157, 167, 189 Brominated flame retardants: congeners of the penta-mix, octa-mix, and deca-mix PBDE formulations; hexabromocyclododecane, tetrabromobisphenol-A Lindane Organotin compounds	In addition, total hydrocarbon measurements (e.g. using UV-fluorescence spectrometry) may be employed under specific circumstances (e.g. after oil spill or PAH-related point source discharges). In addition to the contaminants already covered by the OSPAR CEMP, there are a number of other compounds from the OSPAR list of Chemicals for Priority Action that should be monitored because of their toxicity and environmental relevance. The list provided is not complete
Biological effects measurements	Biological effect techniques as specified in the OSPAR Guidelines for the Integrated Monitoring and Assessment of Contaminants and their Effects, as in Figures 26.1 and 26.2	Additional opportunities for the inclusion of new methods are likely to emerge through the implementation of MSFD and as science develops. Potential examples include indicators of immunocompetence and embryo malformation
Supporting parameters	Shell length, shell and soft body weight, gender, stage of gonadal maturation, grossly visible anomalies, lesions, parasites, sampling depth, hydrography (temperature, salinity, oxygen content, turbidity), nutrients/eutrophication	In the list, parameters are provided that are known to affect both the biological effects responses and the concentration of contaminants. The data can be of use for normalization
Sampling depth	Subtidal or intertidal mussels can be used. Deployed mussels offshore can be positioned at depths 0–8 m	Intertidal specimens may be subject to greater biomarker variability. Subtidal specimens are less robust post-sampling and effects measurements may be more susceptible to post-sampling stress
Storage and transport of bivalves	Transport of bivalves should be completed within than 24 h. They should be transported in an insulated container at 4°C in a damp atmosphere maintained by absorbent materials (such as seaweed and/or paper towel) wetted with seawater	

Table 26.3. Overview of methods and species for integrated gastropod/organotin monitoring (2007 WKIMON Report, revised)

SUBJECT	PARAMETER	COMMENT
Species	Intertidal species:	
	Nucella lapillus	
	Nassarius reticulatus	
	Littorina littorea	
	Offshore species:	
	Buccinum undatum	
	Neptunea antiqua	
Sex	Females and/or males	
Size range	Size ranges are to be selected in accordance with the JAMP Guidelines	
Sample size	Depending on the parameter measured, according to JAMP Guidelines	All measurements should be done in individual gastropods and pooling should be avoided.
Sampling time and frequency	Sampling for all parameters should be carried out at the same time. Sampling frequency according to JAMP Guidelines	

Sampling location	Sampling for all parameters should be carried out at the same site	For coastal and estuarine waters, sites should be selected based on existing WFD sites (where they are established) and TBT hot spot areas like harbours and major shipping routes (see relevant JAMP Guidelines).
Chemical determinands	Organotin compounds in tissue	Guidelines for chemical measurements in biota will be published shortly in *ICES TIMES* series, and in a technical annex to the JAMP Guidelines
Biological effects measurements	Imposex or intersex (species-dependent endpoints, as in the JAMP Guidelines). *ICES TIMES* document on intersex in *Littorina* provides methodological advice	
Supporting parameters	Shell length, organotin compounds in sediment	

Table 26.4. Environmental parameters for inclusion in monitoring programmes (water; 2007 WKIMON Report, revised)

SUBJECT	PARAMETER	COMMENT
Chemistry	Salinity, nutrients, oxygen	
Chemical determinands	Metals: Hg, Cd, Pb, Cu, Zn PAHs: EPA 16 + naphthalene, phenanthrene, dibenzothiophene and their alkylated derivatives CBs: ICES 7 CBs Brominated flame retardants: congeners of the penta-mix, octa-mix and deca-mix PBDE formulations; hexabromocyclododecane, tetrabromobisphenol-A Lindane Organotin compounds	Consideration should be given to bioavailability. To answer the JAMP question relating to concentrations approaching background or zero, there may be a requirement to measure a broader range of chemicals
Physical	Temperature, content of suspended matter	
Biology	Phyto- and zooplankton	Information might be useful for specific events, such as blooms affecting fish health

Table 26.5. Environmental parameters for inclusion in monitoring programmes (sediment; 2007 WKIMON Report, revised)

SUBJECT	PARAMETER	COMMENT
Chemistry	TOC, water content, Al, Li	Al and Li (or other elements as appropriate to the sediment type) are used for normalization of contaminant concentrations
Chemical determinands	Metals: Hg, Cd, Pb, Cu, Zn PAHs: EPA 16 + naphthalene, phenanthrene, dibenzothiophene and their alkylated derivatives CBs: ICES 7 CBs+ CB77, CB81, CB126, CB169 + CB105, CB114, CB123, CB156, CB157, CB167, CB189. Brominated flame retardants: congeners of the penta-mix, octa-mix and deca-mix PBDE formulations; hexabromocyclododecane, tetrabromobisphenol-A Lindane Organotin compounds	Consideration should be given to bioavailability. To answer the JAMP question relating to concentrations approaching background or zero, there may be a requirement to measure a broader range of chemicals
Physical	Sediment type, particle size, colour, index, information on anthropogenic disturbances, sedimentation rates, current flow rates	Anthropogenic disturbance such as trawling or sand and gravel extraction may affect the sediment structure

27 Technical annex: supporting parameters for biological effects measurements in fish and mussels

Leonard Balk, Concepción Martínez-Gómez, Matt Gubbins, and John Thain

27.1 Measurement of supporting metrics for fish: condition indices, gonadosomatic index, hepatosomatic index, and age

27.1.1 Background

For all biological effect techniques within the OSPAR Joint Assessment and Monitoring Programme (JAMP) and OSPAR integrated strategy, there is a requirement to report supporting parameters, including species, sex, fish length, whole fish weight, liver weight, and gonad size. The measurement of gonad size and liver weight is used to provide an indication of reproductive state, and liver weight may also give an indication of general health and well-being. These measurements are used in indices relating gonad weight to whole body weight [gonadosomatic index (GSI)] and liver weight to whole body weight [liver somatic index (LSI) or hepatosomatic index (HSI)]. Both gonad and liver weight will change markedly throughout the year; for comparative purposes, these seasonal variations must be taken into account for the interpretation of biomarker responses, such as EROD and vitellogenin (Vtg). In addition, the condition factor (CF) is a general indicator for fish condition, similarly the condition index (CI) for mussels.

The ICES Working Group on Biological Effects of Contaminants (WGBEC) recently reviewed the measurement of these metrics and their role and importance in fish monitoring programmes (Table 27.1).

Table 27.1. Summary of supporting parameters required for fish

PARAMETER	MEASUREMENT	COMMENT
Live fish whole body weight	To 0.1 g	Blotted dry
Length of fish	To nearest 1 mm	
Liver weight	To 0.1 g	
Gonad weight	To 0.01 g	In addition, record sex
Gonad length	To nearest 1 mm	In addition, record sex
Age	Conducted on otoliths	All individuals sampled

27.1.2 General overview: organ size and related measurements

Organ sizes constitute a very elementary measurement. The measurements can be performed with a minimum of equipment, and the procedures are easy to undertake. At least for some species, it is possible to analyse these variables on frozen material. With minimal instruction, these measurements can be determined by personnel not regularly involved in biomarker analysis, although it is preferable to use personnel familiar with handling fish and able to perform simple dissection of fish.

Data of this type may be of relevance either in their own right, indicating adverse effects of various kinds where the toxic mechanisms are not fully understood as a result of xenobiotic exposure, and/or partly as a supporting variable to biomarkers conducted at the whole individual, tissue, cellular, and subcellular levels. As for all biomarkers in use today, there is a clear need for quality assurance when these measurements are carried out.

One of the most important measurements in this field may be the development of gonads among female fish. This variable is best expressed as gonad size relative to somatic body weight (GSI) and expressed as a percentage value. The best species to use are those where the gonads of juvenile and immature fish are different from adult fish and where there are distinct differences in the genders. For example, it is much easier when the morphology of the female ovary is a single structure, whereas the male testes are paired bilaterally.

This offers the opportunity to investigate when the fish in relation to size and/or age are sexually immature or adult, or indeed have retarded gonad development (often termed sexually immature—SIM) as compared with normal sexual development. This can be expressed as a percentage of sexually immature females among the adult females, and represents the portion of fish with the extreme low value of the GSI value (usually below ~1%) and they have, therefore, a gonad with no or neglected development.

Analogous to the analysis of gonad size is liver size relative to somatic body weight (LSI or HSI). It may be regarded as a parameter in its own right and also as a supporting variable for other biomarkers, such as EROD.

Furthermore, growth (e.g. g year^{-1}), as shown in Kiceniuk and Khan (1987), McMaster *et al*. (1991), and Ericson *et al*. (1998), as well as the condition factor (CF) are relatively straightforward to determine and may be used as markers for adverse effects resulting from xenobiotic exposure. The measurement of CF has not often been used in short-exposure laboratory experiments; however, field observations over longer periods indicate that it may be a valuable measure for adverse effects (see review by van der Oost *et al*., 2003). Recent investigations related to the fish disease index (ICES, 2011) support this assumption.

During periods of high food intake and also in conjunction with the reproductive cycle, an individual may have a higher gross weight at a particular length. This can be assessed by calculating the coefficient of condition (*K*) or by Fulton's condition factor (Bagenal and Tesch, 1978). This is calculated as follows:

$$K = \text{weight length}^{-3}$$

The CF reflects the nutritional state or "well-being" of an individual fish and is sometimes interpreted as an index of growth rate.

Feeding status in fish may be reflected in the CF, may be important for a number of different responses, and, as such, can be included in biomonitoring investigations.

27.1.3 Gonad size in fish—GSI

The reproductive process constitutes (one of) the most essential health signals for the individual animal and, when missing or impaired, indicates an obvious risk for adverse effect both genetically and for population survival. Therefore, decreased sizes of the gonad, of one or both of the genders, indicate an apparent risk for a reduced reproductive potential.

Gonad size is measured as a percentage of somatic body weight, GSI. It has been demonstrated to be a variable that can be influenced by contaminants in a number of different polluted field studies. It should be underlined that the toxicological response observed for this variable could have originated from a number of different toxicological reasons, such as tissue or cell death to more sophisticated regulatory endocrine mechanisms.

GSI is measured by recording the whole body weight of fish and gonad weight to 2 decimal places:

GSI = (gonad weight × 100)/(total body weight*–gonad weight)
(*subtract stomach content)

Deviation in GSI levels could represent a permanent effect or impairment for the reproductive cycle for one or more years (Janssen *et al.*, 1997; Vallin *et al.*, 1999). Both scenarios will seriously affect reproductive potential. Examples of different pollution gradients where reduced gonads have been observed are in bleached kraft pulp mill effluents (Andersson *et al.*, 1988; Sandström *et al.*, 1988; McMaster *et al.*, 1991; Balk *et al.*, 1993; Förlin *et al.*, 1995), including using chlorine-free processes (Karels *et al.*, 2001) and general pollution (Johnson *et al.*, 1988; Noaksson *et al.*, 2001). Laboratory exposure experiments, where effect on the GSI value have been documented, include petroleum mixtures (Truscott *et al.*, 1983; Kiceniuk and Khan, 1987), specific PAHs (Thomas, 1988; Singh, 1989; Thomas and Budiantara, 1995), PCB mixture (Thomas, 1988), pesticides (Ram and Sathyanesan, 1986; Singh, 1989), and cadmium (Singh, 1989; Pereira *et al.*, 1993).

There is no doubt that xenobiotics can affect gonad size through a number of different toxicological mechanisms. However, as for most biomarkers—a variable that shows a natural (annual) biological cycle—it is essential that normal background values are well known, and that an appropriate control material is used for comparison. For the GSI value it should be pointed out that at certain times of the year gonad development is very rapid and different GSI values are obtained within a period of just a few days or weeks. Analysis of the GSI in these periods should be avoided. Baseline studies are important in order to evaluate suitable periods for this variable (Förlin and Haux, 1990; Larsen *et al.*, 1992).

A state of complete disruption of sexual maturation reflects an extreme situation of low GSI values, for example a state of condition when adult (based on age and/or size) fish are unable to develop from the prepubertal condition to the sexually mature stage. Field observations demonstrating a delay or lack of gonad development have been observed in the following species: burbot (*Lota lota*) on the north coast of Bothnian Bay (Pulliainen *et al.*, 1992), English sole (*Parophrys vetulus*) in generally polluted areas in Puget Sound, USA (Johnson *et al.*, 1988), perch (*Perca fluviatilis*) in the effluent water from pulp and paper mills in Baltic waters (Sandström *et al.*, 1988; Sandström, 1994), as well as white sucker (*Catostomus commersoni*) in corresponding effluents in Ontario, Canada (McMaster *et al.*, 1991). Studies have also shown that perch, roach (*Rutilus rutilus*), and brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump in a Swedish freshwater system show corresponding adverse effects (Noaksson *et al.*, 2001, 2003). Although the above-cited field investigations are not all related to suspected PAH contamination, these kinds of disorders have been created in laboratory experiments using petroleum products and pure naphthalene (Thomas and Budiantara, 1995).

27.1.4 GSI confounding factors

Although GSI measurement is robust and easy to perform, there is a need to characterize and avoid confounding factors. For example, female perch do not naturally spawn every year, and the spawning frequency is affected by water temperature, as indicated in Luksiene *et al.* (2000) and Sandström *et al.* (1995). Moreover, in the closely related yellow perch (*Perca flavescens*), both photoperiod and temperature have been suggested to be of importance (Dabrowski *et al.*, 1996).

Therefore, GSI data should be interpreted with regard to the reproductive cycle for each species under investigation.

27.1.5 Liver size of female and/or male fish—LSI (HSI)

Liver size is measured in relation to somatic body weight. This is known as liver somatic index (LSI* or HSI—see above).

LSI is measured by recording whole body weight of fish and liver weight to two decimal places:

LSI = (liver weight × 100)/(total body weight*–liver weight)
(*subtract stomach content)

LSI may be regarded as a relevant measurement because it has been documented to be affected by contaminants in a number of different polluted field studies, for example in pollution gradients of paper and pulp mill effluents where increased LSI values were observed (Andersson *et al.*, 1988; Lehtinen *et al.*, 1990; Hodson *et al.*, 1992; Kloepper-Sams and Owens, 1993; Huuskonen and Lindström-Seppa, 1995; Förlin *et al.*, 1995), as well as decreased LSI levels as reported by Balk *et al.* (1993) and Förlin *et al.* (1995). Other complex effluents shown to affect liver size in various fish species are: leakage water from public refuse dumps (Noaksson *et al.*, 2001, 2003) and effluent from wastewater treatment plants (Kosmala *et al.*, 1998).

Field situations where PAHs and/or organochlorines are suspected contaminants causing increased liver size in various fish species are documented by Sloff *et al.* (1983), Goksøyr *et al.* (1991a), Beyer *et al.* (1996), Leadly *et al.* (1998), Kirby *et al.* (1999a, 1999b), and Stephensen *et al.* (2000). Laboratory experiments shown to affect liver size among different fish species from exposure to organochlorines have been documented by Adams *et al.* (1990), Newsted and Giesy (1993), Arnold *et al.* (1995), Otto and Moon (1995), Gadagbui and Goksøyr (1996), and Åkerblom *et al.* (2000), and for two-stroke outboard-engine exhaust extract (Tjärnlund *et al.*, 1996) and PAHs (Celander *et al.*, 1994) as well as pesticides (Singh, 1989; Åkerman *et al.*, 2003) and cadmium (Singh, 1989).

27.1.6 LSI confounding factors

Although there is no doubt that xenobiotics could affect liver size as a result of different toxicological mechanisms, it should be emphasized that, as for most biomarkers, control/reference fish should be analysed in close/direct parallel with the exposed site(s). In addition, seasonal variation is observed in different fish species (Koivusaari *et al.*, 1981; Förlin and Haux, 1990; Larsen *et al.*, 1992) and must be taken into account at all times. In addition to the time of the year, factors such as feeding behaviour, gender, maturity, age, size, temperature (George *et al.*, 1990), photoperiod, parasites, among others, need to be taken into consideration. Baseline studies are an important strategy in finally evaluating confounding factors (Balk *et al.*, 1996).

27.1.7 Determination of age

It is essential for the interpretation and assessment of biological effect responses that the age of the fish is known. This is particularly important for effect measurements, such as fish diseases, which may be more prevalent in older fish (Stentiford *et al.*, 2010). Age is assessed by removing the otoliths of each fish sampled and using standard procedures. These vary with species and, sometimes location, and specific guidance should be sought from relevant experts, or ICES. In some species, age may be more easily determined in scales or bone. Ideally, age–size relationship (length and weight) should be known for several populations of fish species for longer periods, because the growth of a fish species may vary in different populations, at different locations, and from year to year .

27.1.8 Interpretation of data

The GSI, LSI (HSI), and condition factor are described here as supporting parameters to assist the interpretation of contaminant-related, biological effect measurements. However, it should be noted that these supporting parameters, in their own right, may be influenced by a number of factors which should be described, if known; these include feeding behaviour, gender, maturity, development stage, age, water temperature, presence of parasitic infections and other disease, location, and seasonality.

27.2 Measurement of supporting metrics for mussel: condition indices

27.2.1 Background

In northern Europe, mussels have their main spawning season in late winter to early spring (e.g. February in the UK). During the onset of reproduction, energy normally used in shell and somatic growth is fully utilized for gametogenesis. This is manifested by a marked increase in flesh weight relative to whole body weight, which increases to a maximum at spawning. Post-spawning, flesh weight relative to whole body weight is at a minimum. As a consequence, flesh weight relative to whole body weight or internal shell volume may be regarded as an index of condition.

For all biological effect techniques within the OSPAR mussel integrated strategy, there is a requirement to report supporting parameters, including mussel length, whole body weight, and condition index.

The supporting parameters required for mussels—at least ten animals per site, usually within a specific size (e.g. 40–45 mm) or similar, depending on availability at the site—are summarized in Table 27.2.

Table 27.2. Summary of supporting parameters required for mussels

PARAMETER	MEASUREMENT	COMMENT
Live whole animal weight	To 0.1 g	Must be on animals taken from full immersion, i.e. including water in body cavity (not gaping). Also, blotted dry
Length of animal/width	To nearest mm	
Wet flesh weight	To nearest 0.1 g	Flesh excised from open shell and drained/blotted dry
Dry flesh weight	To nearest 0.01 g	80°C for 24 h and constant dry weight
Wet shell weight	To nearest 0.01 g	Blotted dry
Dry shell weight	To nearest 0.01 g	80°C for 24 h and constant dry weight
Internal shell volume	To 0.1 ml	Not generally conducted, but provides a very accurate measure of condition

27.2.2 Condition index

Condition indices (CI) based on flesh weight relative to whole weight or shell have been used for several years, both in scientific research and in commercial fisheries, and several methods are available (see Lutz, 1980; Aldrich and Crowley, 1986; Davenport and Chen, 1987). The methods may use wet flesh weight, whole weight, and shell size and/or volume, but these are less sensitive as a result of the difficulty in standardizing the degree of wetness. Indices using dry flesh weight are more accurate, particularly when used in relation to internal shell volume. Examples of condition indices are given below:

CI "A" = 100 × dry weight/whole animal weight
CI "B" = 100 × dry weight/wet flesh weight
CI "C" = 100 × dry weight/internal shell volume
CI "D" = (ratio of shell length : shell width)/dry weight

In general, CI "A" is commonly used for convenience and ease of measurement, but the most accurate assessment of condition is CI "C". Whatever condition index is used, it is high before spawning and lower post-spawning when the animal is in poor condition and the flesh weight is greatly reduced relative to the whole animal weight and the volume of the internal shell cavity (Dix and Fergussen, 1984; Rodhouse *et al*., 1984).

It should be noted that condition indices will vary according to body size (Lutz *et al*., 1980). In addition, other factors, such as the level of parasitic infection (Kent, 1979; Thiessen, 1987) and aerial exposure, can adversely affect the condition of mussels.

28 Technical annex: recommended packages of chemical and biological methods for monitoring on a determinant basis

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28.1 Review of CEMP requirements

This technical annex was prepared by reviewing the chemical determinants listed in the OSPAR Coordinated Environmental Monitoring Programme (CEMP) and pre-CEMP (ASMO, 2007a) and considering the most appropriate chemical analyses and biological effects techniques that could be applied in an integrated fashion to monitor for these compounds in the marine environment.

Some general points concerning integrated monitoring were noted during this process:

- In some cases, the list of contaminants that should be reported under the CEMP (and pre-CEMP) may be insufficient for an integrated approach. In order to aid interpretation of biological effects measurements, an integrated assessment may require data on related contaminants, which would elicit a response on the biological effects components of the methods packages. Determinants additional to those required under the CEMP have, therefore, been added to the packages below.
- It was felt that a fully "integrated" approach to monitoring should include passive sampling of contaminants as part of the package of methods. This will provide information on availability of contaminants in sediments and allow for temporally integrated sampling of contaminants in water. (Guidelines for the application of passive samplers are available from ICES Working Group on Marine Sediments in Relation to Pollution (WGMS).)
- The biological effects techniques applied to these packages of methods are listed either in the ICES Working Group on Biological Effects of Contaminants (WGBEC) recommended techniques list (ICES, 2007c) or form part of the fish and shellfish methods packages proposed in the draft Joint Assessment and Monitoring Programme (JAMP) Guidelines for integrated monitoring and assessment of contaminants and their effects (ASMO, 2007b). The biological effects methods included here are separated into those appropriate to monitoring selected fish species, shellfish (mussels), and bioassays (sediment, water, and *in vitro* tests).
- It should be noted that the biological effects methods listed here are those which may form part of an overall integrated monitoring package and are likely to be affected by the OSPAR priority contaminants in question. Many of the effects measurements listed are "general" biological effects, which are indicative of stress or health status of marine organisms or general toxicity in the sediments and water column. These may be affected by a wide range of contaminants and are not specific to the contaminants in question. Therefore, for each group of substances, the most specific and relevant biological effects techniques have also been highlighted.
- These packages of methods should be considered supplemental to the existing JAMP Guidelines for Contaminant-specific (OSPAR 2003–10) and General (1997–7) Biological Effects Monitoring and the JAMP Guidelines on Contaminants in Biota (OSPAR 1999–2) and Sediment (OSPAR 2002–

16). The JAMP Guidelines provide more detailed background on the biological effects and chemical analysis methods referred to here and the necessary cofactors that should be recorded for these techniques. The packages of methods presented here combine contaminant-specific effects with the general biological effects methods that are likely to respond to the contaminants. They also deal with groups of contaminants not addressed by the contaminant specific guidelines and propose further integration of techniques, such as passive sampling and invertebrate methods for metals.

The priority chemical determinants from the OSPAR CEMP and pre-CEMP are as follows (taken from ASMO, 2007a). (The appendices referred to are CEMP appendices.)

The following components of the CEMP are to be measured on a mandatory basis:

- the heavy metals cadmium, mercury, and lead in biota and sediment (appendix 2);
- the PCB congeners CB 28, CB 52, CB 101, CB 118, CB 138, CB 153, and CB 180 in biota and sediment (appendix 3);
- the PAHs anthracene, benz[*a*]anthracene, benzo[*ghi*]perylene, benzo[*a*]pyrene, chrysene, fluoranthene, ideno[1,2,3-*cd*]pyrene, pyrene, and phenanthrene in biota and sediment (appendix 4);
- TBT in sediment (biota voluntary/pre-CEMP; appendix 5).

The following components are currently part of the pre-CEMP and are to be measured on a voluntary basis:

- the brominated flame retardants HBCD and PBDEs 28, 47, 66, 85, 99, 100, 153, 154, and 183 in biota and sediment, and BDE 209 in sediment (appendix 8);
- the planar PCB congeners CB 77, 126, and 169 in biota. Monitoring of those congeners in sediment should be undertaken only if levels of marker PCBs are e.g. 100-fold higher than the background assessment concentration (appendix 9);
- the alkylated PAHs C1-, C2-, and C3-naphthalenes, C1-, C2-, and C3-phenanthrenes, and C1-, C2-, and C3-dibenzothiophenes and the parent compound dibenzothiophene in biota and sediment (appendix 10);
- perfluorooctane sulfonate (PFOS) in sediment, biota, and water (appendix 12);
- polychlorinated dibenzodioxins and furans in biota and sediment (appendix 13).

28.2 Methods package for metals

Although cadmium, mercury, and lead are the only mandatory metal determinants under the CEMP, other metal species are needed to interpret the biological effects data as part of an integrated package. Additional metal species needed include copper and zinc. Metals analysis should be performed on sediments and biota collected from the same times and locations, where possible. Cofactors for sediment analysis are also required, including aluminium and lithium. Diffusive gradients in thin films (DGTs) present the opportunity to undertake passive sampling for metal species to allow temporally integrated sampling of water and measure availability of metals in sediments.

Metal-"specific" biological effects measurements include metallothionein, ALA-D, and oxidative stress, although both metallothionein and oxidative-stress responses are known to be affected by other contaminants. ALA-D is lead-specific and can be measured in fish blood, although it has limited use/expertise across the ICES/OSPAR community. It is recommended that it be applied only in areas where lead contamination is perceived to be a problem or where chemical monitoring indicates that concentrations are significantly above background.

ALA-D is relevant only for fish. Metallothionein can be applied to fish liver and mussel digestive glands, although best results are obtained from mussels. There are a number of oxidative-stress measurements that can be made in both fish and mussels which could add value to an integrated package of metals methods, but owing to the lack of standardized methods, quality assurance, and assessment criteria, it is suggested that this method is not an essential part of the metals package.

A number of "general" biological effects measurements in fish and shellfish will be affected by environmental metal contamination and these are shown in Figure 28.1. *In vivo* bioassays are also relevant measurements for the effects of metals.

Metallothionein in mussels and ALA-D in fish are considered the most specific/relevant biological effects methods for metals.

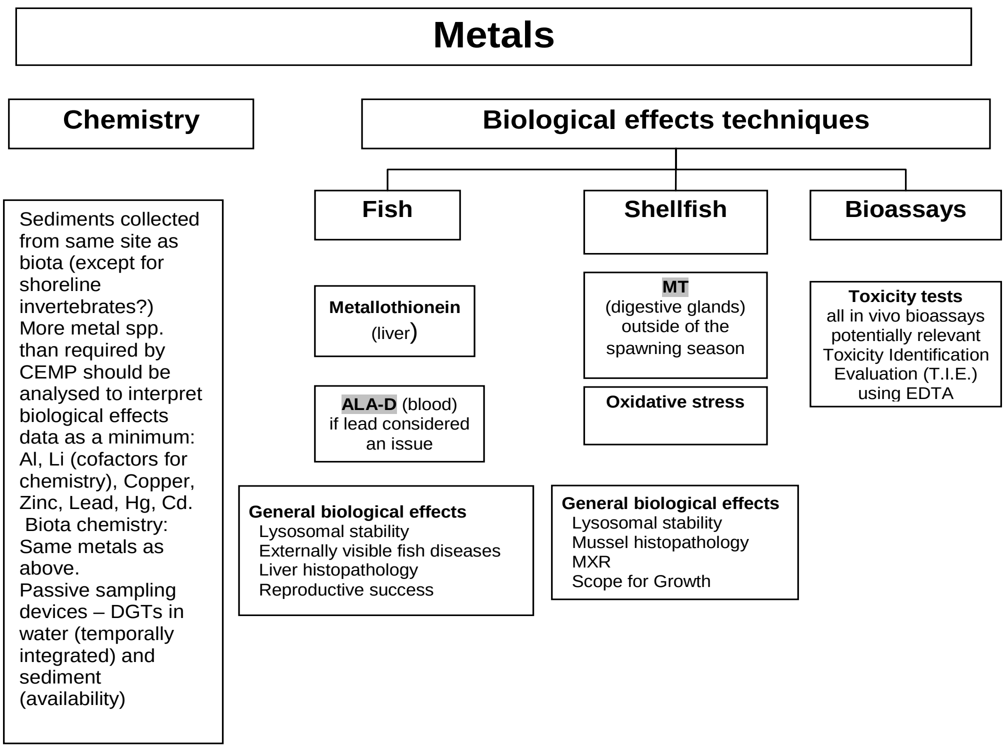

Figure 28.1. Package of chemical and biological effects methods relevant to monitoring for metals. The most specific/relevant biological effects methods are highlighted (bold, shade).

28.3 Methods package for PCBs, polychlorinated dibenzodioxins, and furans

Because of the similarity of their toxicological effects, a single methods package was proposed for polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins, and furans. In addition to the OSPAR CEMP required determinants, additional chlorinated biphenyls (CBs) may cause biological effects, and their analysis should be included in an integrated monitoring approach. These include coplanar CBs CB105, and CB156. A variety of passive sampling devices (e.g. silicone rubber) offer the potential for temporally integrated sampling of these compounds from water and investigation of their availability in sediments, and these should be employed where possible.

There are no truly specific biological effects measurements available for PCBs, polychlorinated dibenzodioxins, and furans. The most relevant are considered to be induction of CYP1A/EROD activity in fish liver and application of the dioxin receptor-based *in vitro* test, DR-CALUX.

Several other general biological effects measurements in fish and shellfish may respond to exposure to these compounds and are shown in Figure 28.2. DR-CALUX is considered the most useful *in vitro* bioassay technique, although chronic *in vivo* bioassays may also be relevant.

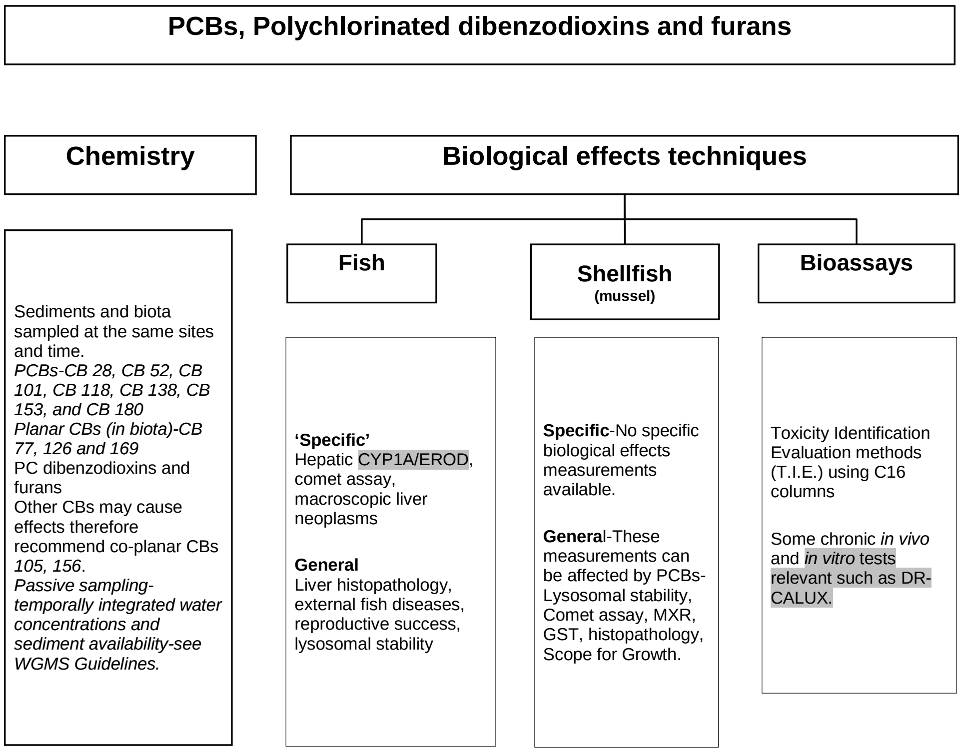

Figure 28.2. Package of chemical and biological effects methods relevant to monitoring for PCBs, polychlorinated dibenzodioxins, and furans. The most specific/relevant biological effects methods are highlighted (bold, shade).

28.4 Methods package for PAHs and alkylated PAHs

Because of their similar toxicological effects, a single package of methods is proposed for polycyclic aromatic hydrocarbons (PAHs) and alkylated PAHs (Figure 28.3). The package of methods is similar to Figure 28.2 above, although chemical determinants should be analysed in sediment and shellfish only for biota. Because of their rapid metabolism in finfish, PAHs should be analysed as metabolites in bile rather than as parent compounds in liver or flesh. As above, passive sampling should also be applied where possible.

Additional specific biological effects are applicable for PAHs/alkylated PAHs. These include PAH metabolites in fish bile and DNA adducts in fish liver. The most relevant/specific biological effects techniques are highlighted as induction of hepatic CYP1A/EROD, DNA adducts, and the DR-CALUX *in vitro* bioassay.

General biological effects measurements will also respond to exposure to these compounds and are given in Figure 28.3.

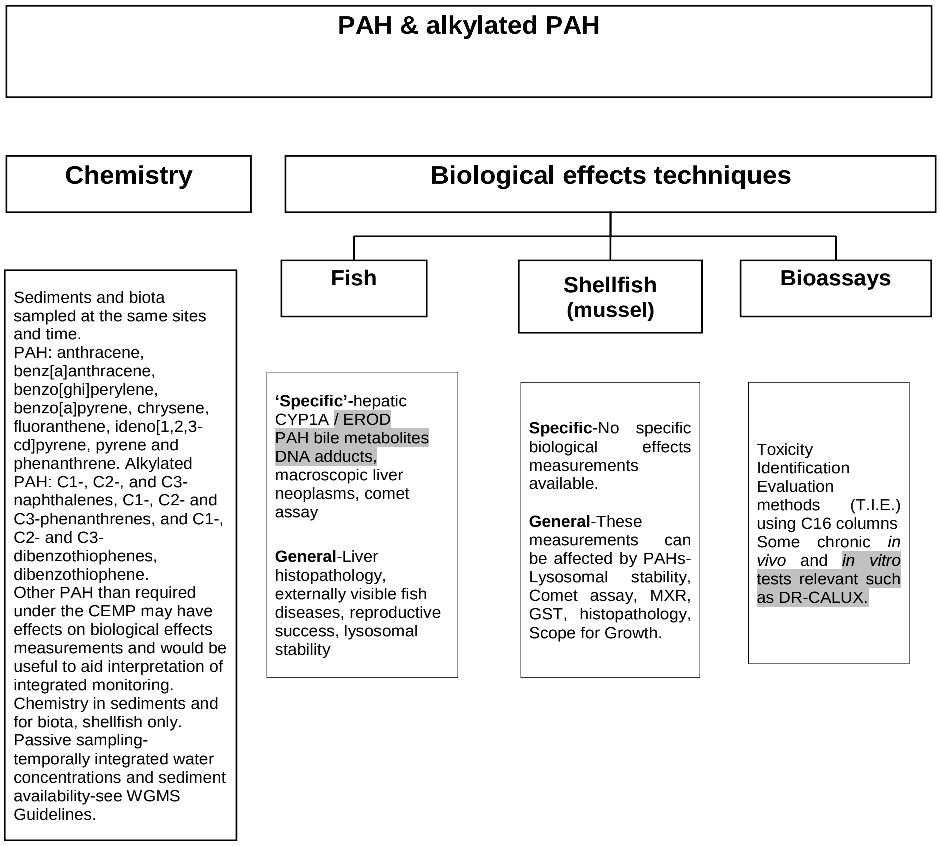

Figure 28.3. Package of chemical and biological effects methods relevant to monitoring for PAHs and alkylated PAHs. The most specific/relevant biological effects methods are highlighted (bold, shade).

28.5 Organotins

It was felt that the package of methods appropriate to organotin monitoring was already very well described by the JAMP Guidelines on organotin-specific monitoring and included a suite of parameters relevant to imposex/intersex in gastropods, TBT, DBT, MBT, TPhT, DPhT, MPhT in sediments (for offshore monitoring), and in biota, where appropriate (voluntary). It was noted that passive sampling for organotins may become an option for integrated monitoring of organotins in future. It was also noted that bivalve embryo bioassays are sensitive to dissolved TBT at the ng l^{-1} level.

28.6 BFRs

It was noted that there are currently very few biological effects methods available and tested in a monitoring context for measuring the effects of brominated flame retardants (BFRs). The determinants required for CEMP are HBCD and PBDEs 28, 47, 66, 85, 99, 100, 153, 154, and 183 in biota and sediment, and BDE 209 in sediment. Passive sampling is also relevant.

There are no specific biological effects techniques available. Thyroid hormone receptor assays in fish blood are relevant, but have not been well field-tested, nor is this an ICES recommended technique. Recent studies on the toxicological properties of these compounds in fish suggest that there are limited overt effects that can be detected by existing techniques.

28.7 PFOS

Perfluorooctane sulfonate (PFOS) analysis in sediment, biota, and water is included in the list of pre-CEMP determinants; however, no specific biological effects techniques are recommended here. It was noted that the compound may have endocrine-disrupting effects and that some endocrine disruptor-relevant endpoints may be appropriate along with general biological effect measurements, such as reproductive success. A battery of short-term, low-volume bioassays (*in vitro* and *in vivo*) using extracts can be used to perform a first screening/assessment of unintended impacts and novel contaminants (see background document on water bioassays). These extracts can be derived from water, sediment, biota, and/or passive samplers. Information obtained from bioanalysis can also be used as input for the design of future monitoring programmes and the development of appropriate higher level biological effects techniques biomarkers. However, a package of methods relevant to PFOS would require further consideration.

29 Discussion document: survey design for integrated chemical and biological effects monitoring

Werner Wosniok and Ian M. Davies

29.1 Background

The joint OSPAR/ICES Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open-Sea Areas (WKIMON) was asked to develop a draft technical annex on the survey design for integrated chemical and biological effects monitoring based on work anticipated to be carried out by the ICES Working Group on Statistical Aspects of Environmental Monitoring (WGSAEM). The purpose of the technical annex would be to provide guidance on the selection of representative stations, taking into account requirements under the Water Framework Directive (WFD) and the proposed Marine Strategy Framework Directive (MSFD).

In particular, it was recommended that this work should build on work by WGSAEM 2007 relating to the spatial design of monitoring programmes and should take into account the approach taken by the UK in redesigning their station network.

Sampling for both a single parameter or for integrated monitoring requires definition of sampling positions, sampling times, and the number of cases per sampling. The strategy to do this is essentially the same for both cases, so the general procedure for planning integrated monitoring can follow that for planning single-parameter monitoring. Considerations on these steps are detailed below in Section 29.3.

29.2 Discussions at ICES/OSPAR SGIMC 2010

The WKIMON group was discontinued in 2008 and was replaced by the ICES/OSPAR Study Group on Integrated Monitoring of Contaminants (SGIMC). SGIMC 2010, therefore, reviewed the opportunities to progress the task to develop a technical annex on survey design.

SGIMC 2010 noted that survey design had been discussed only briefly by WGSAEM 2007, but it had concluded that it was not possible to take this item forward during the meeting. WGSAEM had not returned to this topic since 2007, and it was not clear that they would be able to do so in 2010. However, effective survey design is heavily dependent on statistical analysis and advice.

SGIMC 2010 discussed the opportunities for further development of advice on survey design. The UK approach to redesign involved the definition of monitoring and assessment regions, and the application of a stratified random sampling scheme within the regions (see Section 29.3.4). Since 2007, there has been an increased interest in assessment of monitoring data on regional bases, for example the presentation of contaminant (CEMP) monitoring data in the OSPAR QSR 2010 documents. The EU MSFD has assessment regions (and subregions) as a core element of its assessment system. In anticipation of this, the regional conventions (OSPAR, HELCOM, MED POL) are developing proposals for the definition of subregions within their areas.

SGIMC considered, therefore, that the development of survey design, including subregions for monitoring and assessment, was a considerably wider tissue than just integrated chemical and biological effects monitoring in the context of the OSPAR Hazardous Substances Strategy. For the MSFD, it will be necessary to develop coherent and efficient monitoring programmes for a wide range of descriptors of

"Good Environmental Status". SGIMC, therefore, agreed that the overall task was too large for them to address, but that it was possible for the group to offer comment and advice on aspects of the statistical considerations that will be part of the wider programme of OSPAR to assist in the implementation of MSFD, specifically in the Northeast Atlantic.

In the light of the current implementation for MSFD and the availability of statistical advice from the relevant environmental working groups in ICES, SGIMC is of the opinion that it is not possible to progress this question of survey design further at this time. SGIMC recommends that this discussion document be forwarded to OSPAR as a contribution to the wider survey design task in relation to harmonization of OSPAR and MSFD programmes.

29.3 Some statistical considerations in integrated monitoring

The choice of sampling positions aims at obtaining a sample which reflects the variation of a parameter in the area of interest (i.e. to establish geographic representativeness). This section describes two alternative sampling strategies, adopting either fixed sampling positions or a stratified random position sampling. The stratified random sampling scheme starts from the assumption that there are homogeneous regions from which samples may be taken at random positions. Fixed position sampling avoids problems that could arise from heterogeneity that was not anticipated, which could be a problem for stratified random sampling schemes. On the other hand, a fixed station may, by bad luck, be located at an inappropriate position, but will be reused provided that the monitoring programme continues. With random sampling, such a continuously bad positioning is unlikely, instead it can be expected that "good" and "bad" positions compensate each other in the long run. These considerations apply to selecting positions for monitoring for a single quantity as well as for an integrated plan. They apply similarly to the choice of sampling times, although the latter is also driven by other considerations (e.g. inside/outside the spawning period).

29.3.1 Survey design: general

Survey design is driven by the objectives of the sampling, which are to (WKIMON III report, p. 170):

- assess status (existing level of marine contamination and its effect) and trends across the OSPAR maritime area;
- assess the effectiveness of measures taken for the reduction of marine contamination;
- assess harm (unintended/unacceptable biological responses) to living resources and marine life;
- identify areas of serious concern/hot spots and elucidate their underlying causes;
- identify unforeseen impacts and new areas of concern;
- create the background to develop prediction of expected effects and the verification thereof (hindcasting); and
- direct future monitoring programmes.

Of course, each choice of sampling points and sample sizes for a survey leads to some data on marine contamination and possible effects (provided that anything at all is measured). However, if the survey is expected to generate statements like:

- an assessment of an absolute level ("level at position A is below/above a critical value"), or
- a spatial comparison ("level at position A is lower than / comparable with / higher than at position B), or
- a temporal comparison ("level at position A at time T1 was lower than / comparable with / higher than the level at this position at time T2"), or
- the level of a parameter has changed in part X of the OSPAR maritime area,

with a defined precision, it is necessary to appropriately organize the survey with respect to sample sizes and sampling positions. The aim is to find a survey design that is optimal in the sense that, with a prespecified effort, the most precise map of the spatial parameter distribution is obtained or that a prespecified precision is achieved with the smallest possible effort. To this end, various specifications are needed as input to the survey design, as given in Table 29.1. If the required specifications cannot be given, no *a priori* statement about the quality of the sampling can be made. In this case, a pragmatic way of designing the survey has to be followed, as indicated in the previous section. Then, however, an *a posteriori* determination of the statistical power of the monitoring scheme should be performed to obtain a quantification of the monitoring quality. This should also be done if the optimal design were formally determined, but could not be followed in reality because of practical restrictions.

Table 29.1. Specifications needed as input to the derivation of an optimal survey design

d, the change of biological interest	Numerical specification of the change in parameter level that, if present,is to be detected with safety β. Must be specified for each parameter	No standard
1–β, the power of test procedures	Probability that an existing change at least as large as *d* is detected	90 or 95%
s_a, the analytical error of the biological/chemical analysis procedure	Obtained from analytical experience, e.g. multiple measurements of the same sample	No standard
s_b, the biological variation	Obtained from earlier investigation	No standard
D, the geographical area of interest		No standard
F, an initial guess of the spatial distribution of the parameter of interest	May be taken from pilot investigations or derived as educated guess	If no other information, assume uniform spatial distribution

29.3.2 Survey design: optimal design for fixed stations

An optimal survey design can only be developed in an iterative fashion. Prior to each campaign, an optimal design for that campaign is found by the procedure below. The results obtained from this campaign serve as input information for the optimization of the subsequent campaign.

Assuming that monitoring in a large area is intended, and that *a priori* information on the geographical distribution of the quantity under study is available, the following procedure can be used to derive an initial survey design (size and positions) for a monitoring, according to the first part of the first bullet point.

Step 1. Define *D*, the geographical area of interest (for which the assessment shall be valid; see Section 29.3.1).

Step 2. Determine the necessary number of replicates per sampling location (need knowledge of the sampling variability (analytical + biological, e.g. s_a, s_b), precision requirement plus standard statistics).

Step 3. Take the existing information *F* about the parameter of interest in this area and generate a map of the parameter level over the area of interest (use a standard geostatistical technique). Subdivide the range of the parameter into "iso-concentration" ranges. Find the corresponding "iso-concentration" areas on the map. If an iso-concentration area is ring-shaped, subdivide the ring into at least four sections (e.g. according to compass directions). Ring sections and the non-ring iso-concentration areas define the "sampling cells" addressed below.

Step 4. Define samplings points that are of basic interest or required for formal reasons. These points will not be changed by the following steps.

Step 5. Define an initial number of sampling points (a guess), additional to those from Step 4.

Step 6. Allocate sampling points from Step 5 to initial positions, starting with the geographical means of the sampling cells from Step 3. Define a grid of further candidate positions.

Step 7. For all present sampling points (initially those from Steps 4 and 5), calculate the estimated parameter value from the map of Step 3.

Step 8. Compare the map predictions from Step 3 and Step 7, e.g. by computing the integrated mean square error (IMSE) to characterize the present survey design. Record the IMSE.

Step 9. If there still are unvisited candidate grid locations, change the geographic locations of the free sampling positions to the next grid position (one change per step) and continue with Step 7. Otherwise finish.

The optimal survey design will then be the design that produced the smallest IMSE (e.g. the predictions that best reproduce the initial information). If this IMSE is considered too large, the number of sampling positions has to be increased and Steps 4–9, possibly 3–9, are repeated until a satisfactory result is achieved.

29.3.3 Survey design: a first approach for a fixed-station design

The procedure above may, for various reasons, not be acceptable when designing a monitoring scheme. As an alternative, a simple rule is proposed below.

- Determine the necessary sampling size per sampling position according to precision requirements, as above.
- Use at least three sampling positions. Select these so that they include an unimpacted, a heavily impacted, and an intermediate situation.
- If more than three sample positions are used, their positions should again cover the whole range of parameter values, preferably along a gradient.

The rationale behind this proposal is that it is necessary to obtain information about the best and the worst situations. The extremes are more likely to exhibit changes in future monitoring campaigns than sampling positions with a mean level.

No attempt should be made to generalize the findings from as few as three sampling positions to a large map. The quality achieved by the chosen design should be investigated by an *a posteriori* power analysis.

29.3.4 UK approach to survey redesign

The UK approach to redesigning its station network moves away from site-specific monitoring of hazardous substances to a more regional approach and uses random stratified sediment sampling to inform on status and trends supplemented by a *minimum* of one fish sampling site per region (contained within one stratum) to inform on status and to provide supporting information for biological effects monitoring.

Regions and strata have been defined covering the UK continental shelf. Figure 29.1 shows an example of this, for the region defined as Humber/Wash. The region consists of several strata, which include Water Framework Directive water bodies in the 0–1 nautical mile limit, an intermediate stratum 1–12 nautical miles and two open-sea strata, NE open sea and S open sea.

Collecting all samples at the same time and place may be considered to be the "ideal" survey/sampling strategy for integrated monitoring; however, this is usually not achievable in practice because of the seasonal limitations of some parameters, mobility of fish, unsuitable sediment types, etc. and such "snap-shot" sampling often fails to control local temporal and spatial variation in contaminant concentrations.

A regional (stratified) approach generates more useful management information and can improve the power of the programme to detect trends by controlling local spatial variation.

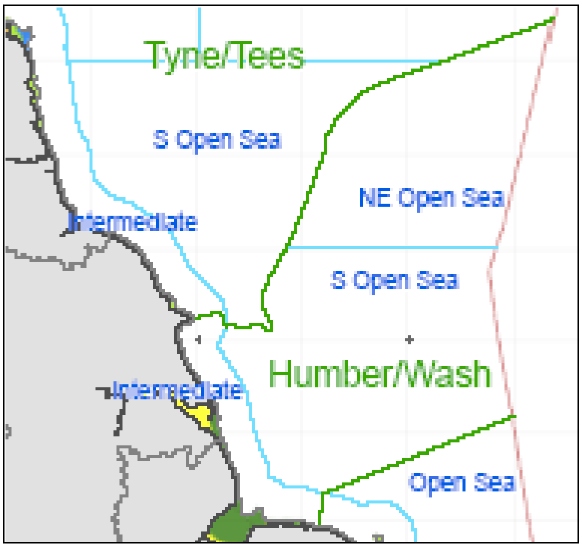

Figure 29.1. An example of the UK regional approach to redesigning the national monitoring station network.

29.3.5 Sample size for integrated monitoring

JAMP Guidelines specify sample sizes for each parameter, although without explicit justification in terms of error probabilities and detectable effects. The sizes given by JAMP seem mainly to be guided by practical considerations (obtain enough material for analysis and consider the time available to collect it). Additional to these considerations, a formal sample size calculation for single parameters could be done by using BAC, EAC, knowledge of analytical errors, and standard specifications for acceptable error probabilities in order to ensure that categorizations of parameters (e.g. a traffic light scheme) are made with defined precision.

Sample size calculation for integrated assessment starts from the integrative assessment criterion, the value of which is to be determined with a specified precision. To calculate the necessary sample size needed for this precision, the mathematical form by which information on single parameters is accumulated into the integrative criterion is exploited, as the statistical distribution of the integrative criterion is determined by the random variation in the single parameters. As an example, the probability of a single parameter in a reference area exceeding its BAC is by definition 10%. In practice, real-world observations from a reference area will not show an exact 10% rate of exceeding values (false positive rate) because of random biological variation and analytical imprecision. In an integrated assessment, each contributing single parameter also contributes a random error, which propagates to the integrative quantity according to the mathematical form by which the integrative quantity is calculated. The distribution of errors in the integrative quantity induced by the single-parameter errors would be used for sample size calculation in the usual way. However, at present, such a mathematical form is not yet available.

30 Technical annex: assessment criteria for biological effects measurements

Ian M. Davies, Matt Gubbins, Ketil Hylland, John Thain, Thomas Maes, Concepción Martínez-Gómez, Michelle Giltrap, Thierry Burgeot, Werner Wosniok, Thomas Lang, and Dick Vethaak

Table 30.1. Assessment criteria for biological effects measurements. Values are given for both background assessment criteria (BAC) and environmental assessment criteria (EAC), as available

Biological effect	**Applicable to:**	**BAC**	**EAC**
Vtg in plasma; µg ml^{-1}	Cod	0.23	
	Flounder	0.13	
Reproduction in eelpout; mean frequency (%)	Malformed larvae	1	
	Late dead larvae	2	
	Growth-retarded larvae	4	
	Frequency of broods with malformed larvae	5	
	Frequency of broods with late dead larvae	5	
EROD; pmol mg^{-1} protein pmol min^{-1} mg^{-1} protein S9 *pmol min^{-1} mg^{-1} microsomal protein	Dab (F)	178	
	Dab (M)	147	
	Dab (M/F)	680*	
	Flounder (M)	24	
	Plaice (M)	9.5	
	Cod (M/F)	145*	
	Plaice (M/F)	255*	
	Four spotted megrim (M/F)	13*	
	Dragonet (M/F)	202*	
	Red mullet (M)	208	
PAHs bile metabolites; *1-OH pyrene **1-OH phenanthrene	Dab	16 ng ml^{-1}; HPLC-F*	
		3.7 ng ml^{-1}; HPLC-F**	
		0.15 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm	22 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm
	Cod	21 ng ml^{-1}; HPLC-F*	483 ng g^{-1} GC-MS*
		2.7 ng ml^{-1}; HPLC-F**	528 ng g^{-1} GC-MS**
		1.1 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm	35 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm
	Flounder	16 ng ml^{-1}; HPLC-F*	
		3.7 ng ml^{-1}; HPLC-F**	
		1.3 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm	29 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm
	Haddock	13 ng ml^{-1}; HPLC-F*	
		0.8 ng ml^{-1}; HPLC-F**	
		1.9 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm	35 pyrene-type µg ml^{-1}; synchronous scan fluorescence 341/383 nm
DR-Luc; ng TEQ kg^{-1} dry wt,	Sediment (extracts)	10	40

silica clean-up			
DNA adducts; nm adducts mol DNA	Dab	1	6
	Flounder	1	6
	Cod	1.6	6
	Haddock	3.0	6
Bioassays; % mortality	Sediment, *Corophium*	30	60
	Sediment, *Arenicola*	10	50
	Water, copepod	10	50
Bioassays; % abnormality	Water, oyster, and mussel embryo	20	50
	Water, sea urchin embryo	10	50
Bioassay; % growth	Water, sea urchin embryo	30	50
Lysosomal stability; min	Cytochemical; all species	20	10
	Neutral red retention: all species	120	50
Micronuclei; 0/00 (frequency of micronucleated cells) [*]Gill cells [**]Haemocytes [+]Erythrocytes	*Mytilus edulis*	2.5[*]	
		2.5[**]	
	Mytilus galloprovincialis	3.9[**]	
	Mytilus trossulus	4.5[**]	
	Flounder	0.0-0.3[+]	
	Dab	0.5[+]	
	Zoarces viviparus	0.3-0.4[+]	
	Cod	0.4[+]	
	Red mullet	0.3[+]	
Comet assay; % DNA tail	*Mytilus edulis*	10	
	Dab	5	
	Cod	5	
Stress on stress; days	*Mytilus sp.*	10	5
AChE activity; nmol min^{-1} mg^{-1} protein [1]Gills [2]Muscle tissue [3]Brain tissue [*]French Atlantic waters [**]Portuguese Atlantic waters [+]French Mediterranean Waters [++]Spanish Mediterranean Waters	*Mytilus edulis*	30[1*]	21[1*]
		26[1**]	19[1**]
	Mytilus galloprovincialis	29[1+]	20[1+]
		15[1++]	10[1++]
	Flounder	235[2*]	165[2*]
	Dab	150[2*]	105[2*]
	Red mullet	155[2+]	109[2+]
		75[3++]	52[3++]
Externally visible diseases*** Ep,Ly,Ul Ep,Ly,Ul Ac,Ep,Fi,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Fi,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Hp,Le,Ly,St,Ul,Xc Ac,Ep,Hp,Le,Ly,St,Ul,Xc Italics: ungraded, bold: graded	Dab	FDI: F: *4.4*, 1.8 M:*5.2*, 2.2 F: *7.0*, 3.1 M: *10.4*, 4.6 F: *6.2*, 2.8 M: *9.5*, 4.3 M: males F: females	FDI: F: *13.9*, 6.6 M: *32.8*, 17.3 F: *17.8*, 7.8 M: *29.8*, 13.3 F: *16.0*, 7.4 M: *26.5*, 12.4
Liver histopathology-non-specific	Dab	NA	Statistically significant increase in mean FDI level in the assessment period compared with a prior observation period or statistically significant upward trend in mean FDI level in the assessment period
Liver histopathology-	Dab	Mean FDI <2	Mean FDI ≥2 A value of

contaminant-specific			FDI = 2 is, e.g. reached if the prevalence of liver tumours is 2% (e.g. one specimen out of a sample of 50 specimens is affected by a liver tumour). Levels of FDI ≥2 can be reached if more fish are affected or if combinations of other toxicopathic lesions occur
Macroscopic liver neoplasms	Dab	Mean FDI <2	Mean FDI ≥2 A value of FDI = 2 is reached if the prevalence of liver tumours (benign or malignant) is 2% (e.g. one specimen out of a sample of 50 specimens is affected by a liver tumour). If more fish are affected, the value is FDI >2
Intersex in fish; % prevalence	Dab	5	
	Flounder		
	Cod		
	Red mullet		
	Zoarces viviaprus		
Scope for growth Joules/h g^{-1} dry wt.	Mussel (*Mytilus* sp.; provisional, further validation required)	15	5
Hepatic metallothionein µg g^{-1} (ww) [1]Whole animal [2]Digestive gland [3]Gills *Differential pulse polarography	*Mussel edulis*	0.6[1*] 2.0[2*] 0.6[3*]	
	Mytilus galloprovincialis	2.01[*] 3.9[2*] 0.6[3*]	
Histopathology in mussels	VVbas: Cell type composition of digestive gland epithelium; µm³ µm⁻³ (quantitative)	0.12	0.18
	MLR/MET: Digestive tubule epithelial atrophy and thinning; µm $µm^{-1}$ (quantitative)	0.7	1.6
	VVLYS and lysosomal enlargement; $µm^3$ $µm^{-3}$ (quantitative)	VvLYS 0.0002	V>0.0004
	S/VLYS: $µm^2$ $µm^{-3}$	4	
	Digestive tubule epithelial atrophy and thinning (semi-quantitative)	Stage ≤1	Stage 4
	Inflammation (semi-quantitative)	Stage ≤1	Stage 3
Imposex/intersex in snails	Gastropod molluscs	See OSPAR adopted criteria	See OSPAR adopted criteria

[***]Assessment criteria for the assessment of the fish disease index (FDI) for externally visible diseases in common dab (*Limanda limanda*).

Ac, *Acanthochondria cornuta*; Ep, epidermal hyperplasia/papilloma; Fi, acute/healing fin rot/erosion; Hp, hyperpigmentation; Le, *Lepeophtheirus* sp.; Ly, lymphocystis; St, *Stephanostomum baccatum*; Ul, acute/healing skin ulcerations; Xc, X-cell gill disease.

Full details of the assessment criteria and how they were derived can be found in the SGIMC 2010, SGIMC 2011, and WKIMON 2009 reports on the ICES website and in the OSPAR background documents for individual biological effects methods.

Data for biomarkers in some northern fish species have been obtained through the IRIS BioSea JIP programme (funded by Total E and P Norge and EniNorge) and the Biomarker Bridges programme (funded by Research Council of Norway) and have been used to develop EAC and BAC values for Arctic fish.

31 References

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Annex 1: Integrated assessment framework for contaminants and biological effects

The development of a framework with which to assess contaminant and biological effects data together is essential to the delivery of integrated monitoring and assessment. A multistep process is proposed which follows on from experience of the assessment of contaminants data for sediment, fish, and shellfish in OSPAR contexts. The process is informed initially by the individual assessment of determinands (contaminants or effects) in specific matrices at individual sites against the defined assessment criteria (BAC and EAC). Such assessment criteria for biological effects have been developed over recent years and are included in OSPAR background documents, and for contaminants have been used by OSPAR groups, for example, in the QSR 2010. Initial comparisons determine whether the determinand and site combinations are < BAC (blue), between the BAC and EAC (green), or > EAC (red). This summarized indicator of status for each determinand can then be integrated over a number of levels: matrix (sediment, water, fish, mussel, gastropod), site, and region and expressed with varying levels of aggregation to graphically represent the proportion of different types of determinands (or for each determinand, sites within a region) exceeding either level of assessment criteria.

Such an approach has several advantages. The integration of data can be simply performed on multiple levels depending on the type of assessment required and the monitoring data available. The representation of the assessment maintains all of the supporting information, and it is easy to identify the causative determinands that may be responsible for exceeding EAC levels. In addition, any stage of the assessment can be readily unpacked to a previous stage to identify either contaminant or effects measurements of potential concern or sites contributing to poor regional assessments.

This approach builds on the OSPAR MON regional assessment tool developed for contaminants. The development of BAC and EAC equivalent assessment criteria for biological effects, which represent the same degree of environmental risk as indicated by BAC and EAC values for contaminants, allows the representation of these monitoring data alongside contaminant data using the same graphical representation approach. The inclusion of biological effects data to the system adds considerable value to the interpretation of assessments. Where sufficient effects monitoring data are available, confidence can be gained that contaminants are not having significant effects even where contaminant monitoring data are lacking. In instances where contaminant concentrations in water/sediment are >EAC, a lack of EAC threshold breach in appropriate effects data can provide some confidence that contaminant concentrations are not giving rise to pollution effects (due, for example, to lack of availability to marine biota). Similarly, the inclusion of effects data in the assessment framework can indicate instances where contaminants are having significant effects on biota, but have not been detected or covered in contaminant-specific chemical monitoring work.

Application to determination of Good Environmental Status for Descriptor 8 of the Marine Strategy Framework Directive

The assessment framework described below provides an appropriate tool for assessment of environmental monitoring data to determine whether or not "Good Environmental Status" is being achieved for Descriptor 8 of the MSFD (concentrations of contaminants are at levels not giving rise to pollution effects). Determinands with EAC or EAC equivalent assessment criteria provide appropriate indicators with quantitative targets. The assessment of contaminant and effects monitoring data against these EAC level assessment criteria provides information both on concentrations of contaminants likely to give rise to effects and the presence/absence of significant effects in marine biota.

Owing to the relatively large number of determinands monitored under the integrated approach, it is inappropriate to adopt an approach whereby EAC level failure of a single determinand results in failure of GES for a site or region. A more appropriate approach would involve the setting of a threshold (%) of proportion of determinands that should be <EAC to achieve GES. Such an approach would avoid the failure of sites or regions as a result of occasional outlying, erroneous results for particular determinands. The setting of an appropriate threshold for overall regional assessment for MSFD will require consideration and revision in the light of testing the framework described here with real monitoring data. However, an initial threshold of 95%<EAC (to ensure that the vast majority, but not all, of contaminants/effects measurements should be <EAC) is proposed here for the purposes of testing the system.

Example application of the integrated assessment framework

In order to best demonstrate how monitoring data (assessed against BAC and EAC) can be integrated for matrices, sites, and regions and ultimately provide an assessment that could be useful for determination of GES for Descriptor 8, a worked example is provided below following a five-step process.

Step 1: Assessment of monitoring data by matrix against BAC and EAC

All determinands available for a specific site assessment are compiled with results presented by monitoring matrix and expressed as a colour depending on whether or not the value exceeds BAC or EAC. In the example provided below (Figure A1), determinands and their status are provided for illustrative purposes only to show how subsequent integration can be performed. A red classification indicates that the EAC is exceeded, blue indicates compliance with the BAC, whereas green indicates concentrations or levels of effects are between the BAC and EAC.

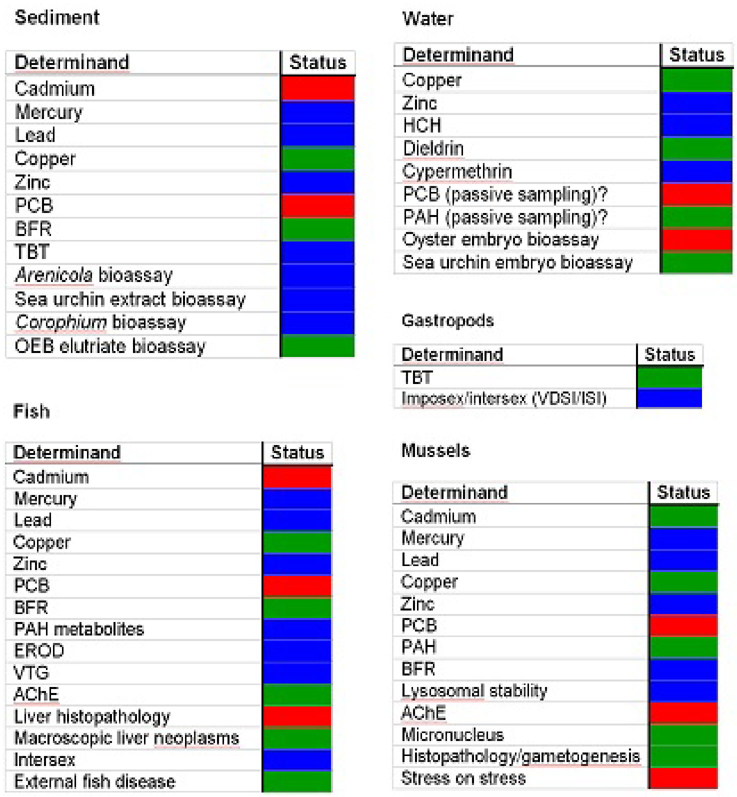

Figure A1. Illustration of classification of measurements of contaminants and their effects using a three colour scheme

Step 2: Integration of determinands by matrix for a given site

For each of the five matrices, the results of the individual determinand assessments are aggregated into categories: contaminants, exposure indicators, effects indicators, and, for sediment/water matrices, also passive sampling and bioassay categories. It is necessary to separate the biological effects measurements into different categories depending on whether or not an EAC-equivalent assessment criterion (AC) has been set. Otherwise, aggregated information on the proportion of determinands exceeding the separate AC will be incorrect. For simplicity, these categories have been termed "exposure indicators" (where an EAC has not been set) and "effects indicators" where an EAC (equivalent to significant pollution effect) has been set for the measurement. On subsequent aggregation/integration of these indicators across matrices for a specific site, bioassays are considered "effects indicators" as EAC are available. It should be possible to include data from passive sampling in both the water and sediment schemes when assessment criteria have become available. They are nominally included in the example here to show how they could be included.

The integration by matrix and category of determinand can be expressed by three-coloured bars showing the proportions of determinands that exceed the BAC and EAC as shown below (Figure A2). Each method for contaminant, effect, or exposure assessment carries the same weight, within the matrix, in the integration shown in Figure A2. Note that for mussels in this instance, no exposure indicators are used, because all of the biological effects measurements have EAC available.

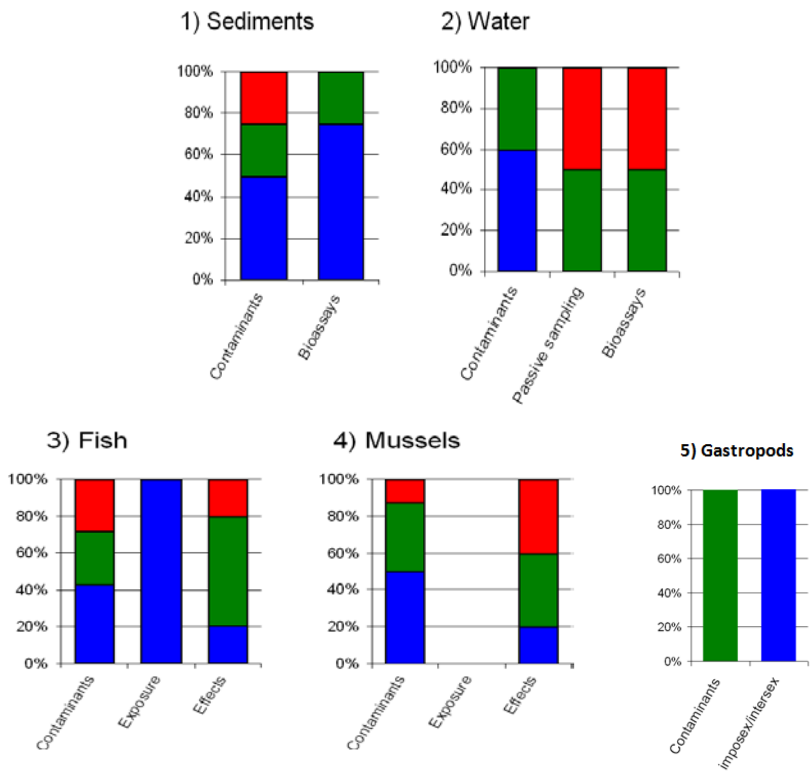

Figure A2. Integration of three colour classifications of measurements of contaminant concentrations and their effects integrated across determinands within matrices.

Step 3: Integration of matrices for a site assessment

In order to simply express the results of assessment for a particular site, information can be aggregated across matrices and expressed by determinand category, as shown below (Figure A3). In order to achieve this, results from passive sampling from sediment and water categories could be integrated into the contaminant indicator graphic and bioassays and gastropod intersex/imposex integrated into ”effects indicators”. Thus, the outcome of assessment of all determinands from all matrices can be expressed for a whole site. In practice, the process adopted is to sum the percentages of each colour in, say, the “contaminants” columns for each matrix in Figure A2, and then to scale the sums to a total of 100%. The results for each matrix, therefore, carry equal weight in the integration shown in Figure A3.

For some assessments, this will be the highest level of aggregation required. However, for assessments covering larger geographical areas (subregional, regional, national, regional seas for the MSFD, etc.) where assessments need to be undertaken across multiple sites, a further level of integration is required (Steps 4 and 5).

For transparency, each determinand grouping is labelled with the matrices from which it is comprised. Thus, it can quickly be determined whether the site assessment is composed of all or just a subset of the monitoring matrices. In the example below (Figure A3), all five matrices have been used to determine the overall site assessment. However, only for fish (matrix 3) were there any effects measurements that did not have EAC available for assessment. Therefore, the exposure indicators graphic is labelled to show that only matrix 3 contributed to the site assessment of indicators of exposure.

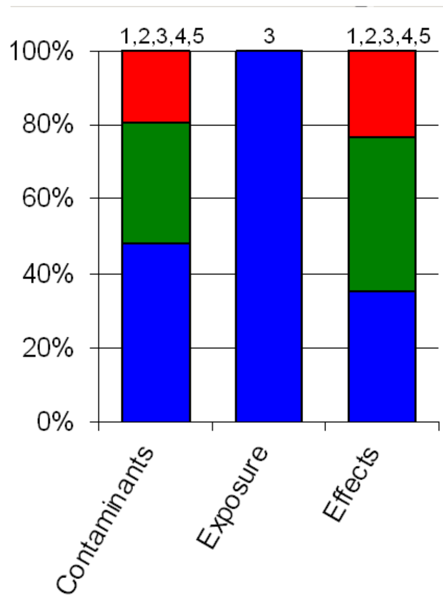

Figure A3. Integration of three colour classifications of measurements of contaminant concentrations and their effects integrated across determinands and matrices.

Step 4: Regional assessment across multiple sites

This can be done at multiple levels (aggregation of data at the subregional, regional, and national levels) in different ways to express both the overall assessment of proportion of determinands (across all matrices) exceeding both assessment thresholds (BAC/EAC; approach A) and by determinand for the region showing the proportion of sites assessed in the region that exceed the thresholds (approach B). Both approaches show the overall proportion of determinand/site incidences of threshold exceedance. However, approach A shows most clearly which determinands are responsible for any EAC exceedance, whereas approach B shows a more aggregated, summarized representation of the same information by determinand category. Both can be constructed directly from the output of Step 1.

Step 4A: Regional assessment of sites by determinand

This shows a graphical representation (Figure A4) of the proportion of sites falling into each status class for each determinand across all relevant matrices (many determinands are only relevant to one or some of the matrices).

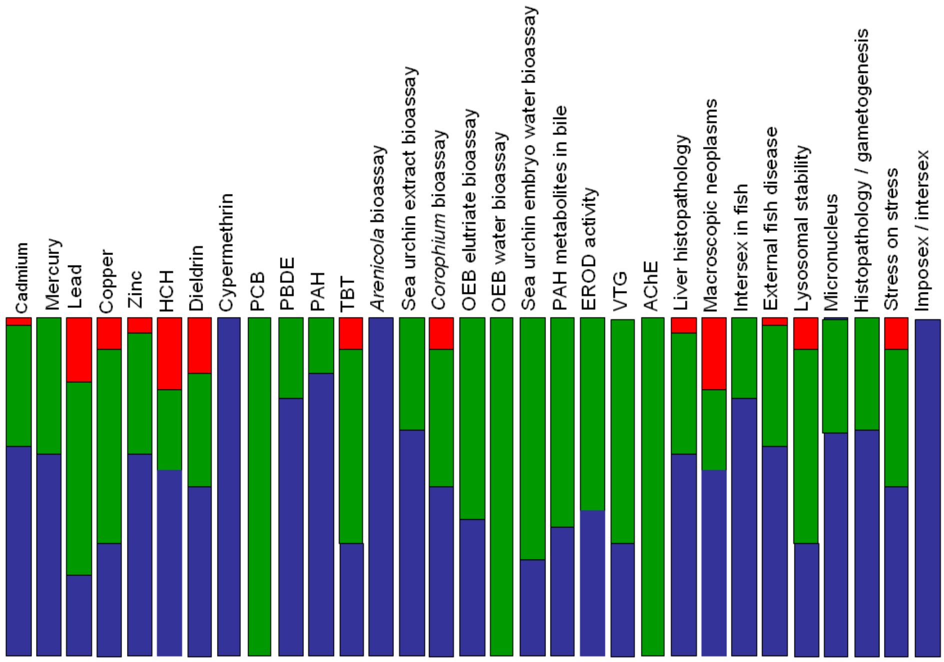

Figure A4. Integration of three colour classifications of measurements of contaminant concentrations and their effects integrated across sampling sites within an assessment region.

Step 4B: Regional assessment of sites by determinand category

The above regional assessment can be summarized by determinand category as was demonstrated in Step 3 for the site assessment and shown below (Figure A5).

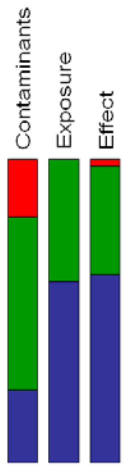

Figure A5. Integration of three colour classifications of measurements of contaminant concentrations and their effects integrated across sampling sites and determinands within an assessment region.

Step 5: Overall assessment

The assessment by region can be aggregated further into a single schematic showing the proportion of all determinands across all sites that exceed BAC and EAC (Figure A6). This can be used for the purposes of an overall assessment, and it is proposed that a simple threshold figure (e.g. 95% <EAC) is used to determine whether or not "Good Environmental Status" for Descriptor 8 is met in this assessment. The overall assessment can be easily unpacked through the steps above to determine which sites

and determinands (effects types or contaminants) are contributing to, for example, the proportion of red (>EAC) data, and thereby potentially leading to failure to achieve GES for a region.

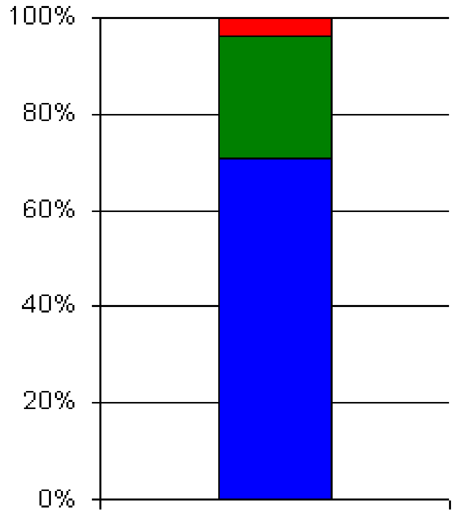

Figure A6. Integration of three colour classifications of measurements of contaminant concentrations and their effects integrated across sampling sites, matrices, and determinands within an assessment region.

Conclusion

An assessment framework has been presented that integrates contaminant and biological effects monitoring data and allows assessments to be made across matrices, sites, and regions. It is simple and transparent and allows for multiple levels of aggregation for different assessment requirements. Such an approach has been used with success for a wide range of contaminants data in the OSPAR QSR 2010 and can be extended to include other chemical and biological effects measurements through the application of a coherent set of assessment criteria. This approach can provide a suitable approach for the assessment of GES for Descriptor 8 of the MSFD. Current research projects and the initial assessment for the MSFD due in 2012 provide opportunities to gain experience in its use.

Annex 2: Fish disease monitoring in the OSPAR Coordinated Environmental Monitoring Programme (CEMP) reflecting ICES advice (ICES, 2005a)

Table A1. PAH-specific biological effects monitoring

	Species	Diseases	Numbers	Guidelines
Macroscopic liver neoplasms	Dab (first priority; *Limanda limanda*)	Macroscopic liver nodules >2 mm in diameter, subsequent quantification of histologically identified liver neoplasms	Size group ≥25 cm: 50 (if not available in sufficient numbers, include size group 20–24 cm)	JAMP Guidelines based on Bucke *et al.* (1996). Relevant in addition: ICES (1989). Feist *et al.* (2004). BEQUALM
	Flounder (*Platichthys flesus*)		Size group ≥30 cm: 50 (if not available in sufficient numbers, include size group 25–29 cm)	
Liver histopathology	Dab (first priority; *Limanda limanda*)	Non-specific lesions, early toxicopathic non-neoplastic lesions, foci of cellular alteration, benign neoplasms, malignant neoplasms	Size group 20–24 cm: 50	JAMP Guidelines based on ICES (1997)
	Flounder (*Platichthys flesus*)		Size group 25–29 cm: 50	Relevant in addition: Feist *et al.* (2004). BEQUALM
	Dragonet (*Callionymus* spp.)		Size group 10–15 cm: 50	No JAMP guidelines so far. Relevant: Feist *et al.* (2004)

Table A2. General biological effects monitoring

	Species	Diseases	Numbers	Guidelines
Externally visible fish diseases	Dab (first priority; *Limanda limanda*)	Lymphocystis Epidermal hyperplasia/papilloma Acute/healing skin ulcers X-cell gill disease Hyperpigmentation	Size group 15–19 cm: 100 Size group 20–24 cm: 100 Size group ≥25 cm: 50	JAMP Guidelines based on Bucke *et al.* (1996). Relevant in addition: ICES (1989). BEQUALM
	Flounder (*Platichthys flesus*)	Lymphocystis Acute/healing skin ulcers	Size group 20–24 cm: 100 Size group 25–29 cm: 100 Size group ≥30 cm: 50	
	Cod (*Gadus morhua*)	Acute/healing skin ulcers Skeletal deformities Pseudobranchial swelling *Cryptocotyle* sp.	Size group ≥29 cm: 100 Size group 30–44 cm: 100 Size group ≥45 cm: 50	
	Whiting (*Merlangius merlangus*)	Epidermal hyperplasia/papilloma *Lernaeocera branchialis* *Diclidophora merlangi* *Clavella adunca*	Size group 15–19 cm: 100 Size group 20–29 cm: 100 Size group ≥30 cm: 50	No JAMP guidelines so far. Relevant: Bucke *et al.* (1996)
Macroscopic liver neoplasms	Dab (first priority; *Limanda limanda*)	Macroscopic liver nodules >2 mm in diameter, subsequent quantification of histologically identified liver neoplasms	Size group ≥25 cm: 50 (if not available in sufficient numbers, include size group 20–24 cm)	JAMP Guidelines based on Bucke *et al.* (1996). Relevant in addition: ICES (1989). Feist *et al.* (2004). BEQUALM
	Flounder (*Platichthys flesus*)		Size group ≥30 cm: 50 (if not available in sufficient numbers, include size group 25–29 cm)	

Liver histopathology	Dab (first priority; *Limanda limanda*)	Non-specific lesions, early toxicopathic non-neoplastic lesions, foci of cellular alteration, benign neoplasms, malignant neoplasms	Size group 20–24 cm: 50	JAMP Guidelines based on Bucke *et al.* (1996). Relevant in addition: ICES (1989). Feist *et al.* (2004). pp. BEQUALM
	Flounder (*Platichthys flesus*)		Size group 25–29 cm: 50	
	Dragonet (*Callionymus* spp.)		Size group 10–15 cm: 50	No JAMP guidelines so far for Dragonet. Relevant: Feist *et al.* (2004)

32 Abbreviations and acronyms

AC	assessment criteria
ACh	acetylcholine
AChE	acetylcholinesterase
ACR	acute–chronic ratio
AhR	aryl hydrocarbon receptor
AR	androgen receptor
ASE	accelerated solvent extraction
BAC	background assessment concentrations or criteria
BC	background concentrations
BEQUALM	Biological Effects Quality Assurance in Monitoring
BRC	background/reference concentrations
CCF	contaminant concentration factors
CEMP	Coordinated Environmental Monitoring Programme
Cf	concentration factor
CF	condition factor
CI	condition index
CSEMP	Clean Seas Environment Monitoring Programme
CYP1A	cytochrome P450 1A
DMBA	9,10-dimethyl 1,2 benzanthracene
DPP	differential pulse polarography
DRZ	diagonal radioactive zones
EAC	environmental assessment criteria
ED	endocrine disruptor
EDA	effects-directed analysis
EDC	endocrine-disrupting chemicals
EDMAR	endocrine disruptors in the marine environment
ELISA	enzyme-linked immunosorbent assay
EMS	ethyl methanosulfate
ER	effect-range
ER-L	effect range–low
ER	oestrogen receptor
ERE	oestrogen-responsive element
ERL	environmental risk limits
EVD	externally visible diseases
FCA	foci of cellular alteration
FDI	fish disease index

FFPE	formalin-fixed, paraffin-embedded
GES	Good Environmental Status
GnRH	gonadotrophin-releasing hormone
GPC	gel permeation chromatography
GSI	gonadosomatic index
GtH	gonadotrophin hormone
HSI	hepato somatic index
HSP	heat-shock proteins
HSS	Hazardous Substances Strategy
IARC	International Agency for Research on Cancer
ICES	International Council for the Exploration of the Sea
IPCS	International Programme on Chemical Safety
JAMP	Joint Assessment and Monitoring Programme
LH	liver histopathology
LMS	lysosomal membrane stability
LOEC	lowest observed effect concentration
LSC	lysosomal structural changes
LSI	liver somatic index
Lv-I	lipovitellin I
MCWG	Marine Chemistry Working Group
MDR	mean diverticular radius
MERMAN	Marine Environment Monitoring and Assessment National
MET	mean epithelial thickness
MFO	mixed-function oxygenase
MLN	macroscopic liver neoplasms
mlR	mean luminal radius
MPE	maximum permissible effect
MPR	maximum permissible risk
MPTW	mean proportion of tubule width
MSFD	Marine Strategy Framework Directive
MT	metallothionein
NE	negligible effect
NOEC	no observed effect concentration
NR	negligible risk
NRC	Nature Research Center
NRR	neutral red retention
OSI	ovotestis severity index
PAC	polycyclic aromatic compounds

PAF	potentially affected fraction
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbon
PBB	polybrominated biphenyls
PCA	Principal component analysis
PCDD	polychlorinated dibenzo-p-dioxin
PNR	per cent net response
Pv	phosvitin
QA	quality assurance
QSAR	quantitative structure–activity relationship
RIA	radioimmunoassay
RPS	radiation protection supervisor
RT-PCR	reverse transcription polymerase chain reaction
SCGE	single-cell gel electrophoresis
SE	serious effect
SET	sea-urchin embryo test
SETAC	Society of Environmental Toxicology and Chemistry
SFG	scope for growth
SGIMC	Study Group on Integrated Monitoring of Contaminants
SIME	substances in the marine environment
SOP	standard operating procedure
SoS	stress on stress
SPE	solid phase extraction
SR	serious risk
SSD	species-sensitivity distributions
TEF	toxic equivalency factors
TEQ	toxic equivalent quotient
TIE	toxicity identification evaluation
TIMES	Techniques in Marine Science
TOSC	total oxyradical scavenging capacity
TPAH	total polycyclic aromatic hydrocarbon
TRE	toxicity reduction evaluation
TMM	time to maximum mortality
TU	toxic units
VDSI	vas deferens sequence index
VvBAS	volume density of basophilic cells
Vtg	vitellogenin
WFD	Water Framework Directive

WGBEC	Working Group on Biological Effects of Contaminants
WGPDMO	Working Group on Pathology and Diseases of Marine Organisms
WGSAEM	Working Group on Statistical Aspects of Environmental Monitoring
WHO	World Health Organization
YES	yeast oestrogen screen

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