OSPAR COMMISSION

Background document and technical annexes for biological effects monitoring

Monitoring and Assessment Series

2012

OSPAR Convention

The Convention for the Protection of the Marine Environment of the North-East Atlantic (the “OSPAR Convention”) was opened for signature at the Ministerial Meeting of the former Oslo and Paris Commissions in Paris on 22 September 1992. The Convention entered into force on 25 March 1998. The Contracting Parties are Belgium, Denmark, the European Union, Finland, France, Germany, Iceland, Ireland, Luxembourg, the Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

Convention OSPAR

La Convention pour la protection du milieu marin de l'Atlantique du Nord-Est, dite Convention OSPAR, a été ouverte à la signature à la réunion ministérielle des anciennes Commissions d'Oslo et de Paris, à Paris le 22 septembre 1992. La Convention est entrée en vigueur le 25 mars 1998. Les Parties contractantes sont l'Allemagne, la Belgique, le Danemark, l’Espagne, la Finlande, la France, l’Irlande, l’Islande, le Luxembourg, la Norvège, les Pays-Bas, le Portugal, le Royaume-Uni de Grande Bretagne et d’Irlande du Nord, la Suède, la Suisse et l’Union européenne.

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Many thanks to ICES and the scientific groups whose work made it possible to produce this report.

Editorial note

1. The suite of Background documents, technical annexes and a discussion document on survey design form ICES's advice to a request from the OSPAR Commission relating to the integrated monitoring of chemicals and of their biological effects. They were developed over a period of 3 years.

2. Together with the Integrated JAMP Guidelines for the Integrated Monitoring and Assessment of Contaminants and the background documents and technical annexes for chemical monitoring already adopted by OSPAR, this suite comprises a coherent basis for integrated chemical and biological effects monitoring. The structure of ICES's advice is as follows: 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.

3. The discussion document on survey design for integrated chemical and biological effects monitoring represents the best advice currently available on the subject but would need to be further developed at a later stage. Its aim is to provide guidance on the selection of representative stations, taking into account requirements under the Water Framework Directive and the Marine Strategy Directive, and for the selection of stations for integrated monitoring.

4. The Background Documents on Scope for Growth and on Fish Vitellogenin as a biomarker of exposure to xenoestrogens are not included in this suite as they require further work.

5. This suite provides important information in relation to the on-going processes regarding the Marine Strategy Framework Directive and the preparation of the Initial assessments. As integrated monitoring and assessment continue to evolve it is important for future revisions of techniques and assessment criteria to be harmonized with the MSFD cycle in order to give some stability to assessments.

6. 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 enable 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 identified to add value to the integrated assessment, these have been described as "additional methods" and are not considered essential. **Table a** below gives an overview of the elements featuring in the Integrated JAMP Guidelines for the Integrated Monitoring and Assessment of Contaminants. The recommended set of techniques; i.e the "core methods" are indicated with solid lines and the "additional methods" in broken lines.

7. **Table b** indicates the background documents in Publication 333/2007 being replaced or updated in this publication.

Table a: **Overview of biological effects techniques and corresponding integrated guidelines**.

Elements featuring in the Integrated JAMP Guidelines for the Integrated Monitoring and Assessment of Contaminants (adopted in 2012 on a 3-yr trial basis)					2012 Publication 589	... replaces or updates ...2007 Publication 333
ECOSYSTEM COMPONENTS OF INTEGRATED ASSESSMENT	BIOTA	Fish	Tissue chemistry	Cd, Cu, Zn, Hg, Pb	(see existing JAMP Guidelines)	
				CBs	(see existing JAMP Guidelines)	
				BFRs	(see existing JAMP Guidelines)	
				Fluorinated compounds		
			Subcellular	PAH metabolites	CHAPTER 12	chapter 5
				EROD/CYP1A	CHAPTER 3	chapter 3
				Vitellogenin	*[TO BE FINALISED]*	
				AChE	CHAPTER 1	
				Comet Assay	CHAPTER 2	
				DNA-adducts	CHAPTER 4	chapter 11
				Lysosomal Stability	CHAPTER 9	chapter 4
				Micronuclei	CHAPTER 11	
			Tissue response	Liver histopathology	CHAPTER 5	
				Macroscopic Liver Neoplasms	CHAPTER 5	chapter 6
				Intersex	CHAPTER 7	
			Whole organism	Externally visible Fish Disease	CHAPTER 5	
				Reproductive Success	CHAPTER 13	chapter 12
		Mussel	Tissue chemistry	Cd, Hg, Pb, Cu, Zn	(see existing JAMP Guidelines)	
				PCBs	(see existing JAMP Guidelines)	
				PAH	(see existing JAMP Guidelines)	
				BFRs	(see existing JAMP Guidelines)	
				Fluorinated compounds		
			Subcellular Response	Lysosomal Stability	CHAPTER 9	
				AchE	CHAPTER 1	
				Micronuclei	CHAPTER 11	
				Metallothionein	CHAPTER 10	
				COMET	CHAPTER 2	
			Tissue Response	Histopathology/Gametogenesis	CHAPTER 5	
			Whole Organism	Stress on Stress	-	
				Scope for Growth	*[TO BE FINALISED]*	chapter 14
		Gastropod	Tissue response	Imposex / intersex	(see existing JAMP Guidelines)	
	SEDIMENT	Sediment chemistry		Mandatory CEMP list (JAMP) OSPAR hazardous substances list	(see existing JAMP Guidelines)	
		Sediment characteristics				
		Sediment bioassays		JAMP Guidelines for general biological effects monitoring, Technical Annexes 1, 2 and 3:	(see existing JAMP Guidelines)	
				(1) whole sediment bioassay	CHAPTERS 14 & 15	chapter 10
				(2) sediment pore-water bioassays	CHAPTERS 14 & 15	chapter 8
				(3) sediment sea water elutriates	CHAPTERS 14 & 15	chapter 8
				(4) DR-Luc	CHAPTER 8	
		Benthic fauna		Benthic community indices		
	WATER	Water chemistry		Mandatory CEMP list (JAMP)	(water monitoring not covered by JAMP)	
				WFD priority substances	(WFD monitoring guidance)	
		Water extract / passive sampler		Mandatory CEMP list (JAMP)	(e.g. ICES guidance on use passive samplers)	
				WFD priority substances	(WFD monitoring guidance)	
		Hydrography				
		Bioassays		JAMP Guidelines for general biological effects monitoring, Technical Annex 4:	(CHAPTER 14, 15 and 17)	chapter 7
				(1) oyster and mussel embryo		chapter 8
				(2) Sea urchin embryo	CHAPTER 14	chapter 8
				(3) Copepods	CHAPTER 15	chapter 9

Table b: List of Background documents in Publication 333/2007 updated or replaced in this publication

Background documents and Technical annexes in Publication 589/2012	New in 589/2012	Chapter updated or replaced in Publication 333/2007	Coverage of JAMP Guidelines General Biological Effects
Chapter 1: Background Document: Acetylcholinesterase assay as a method for assessing neurotoxic effects in aquatic organisms	Yes		
Chapter 2: Background Document: Comet assay as a method for assessing DNA damage in aquatic organisms	Yes		
Chapter 3: Background Document: Cytochrome P4501A activity (EROD)		Chapter 3	Technical annex 5
Chapter 4: Background Document: DNA adducts		Chapter 11	
Chapter 5: Background Document: Externally visible fish diseases, macroscopic liver neoplasms, and liver histopathology		Chapter 6	Technical Annexes 7, 8, 9
Chapter 6: Background Document: Histopathology of mussels (Mytilus spp.) for health assessment in biological effects monitoring	Yes		
Chapter 7: Background Document: Intersex (ovotestis) measurement in marine and estuarine fish	Yes		
Chapter 8: Background Document: In vitro DR-Luc/DR-CALUX® bioassay for screening of dioxin-like compounds in marine and estuarine sediments	Yes		
Chapter 9: Background Document: Lysosomal stability as a global health status indicator in biomonitoring		Chapter 4	Technical Annex 6
Chapter 10: Background Document: Metallothionein (MT) in blue mussels (Mytilus edulis, Mytilus galloprovincialis)	Yes		
Chapter 11: Background Document: Micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms	Yes		
Chapter 12: Background Document: PAH metabolites in bile		Chapter 5	
Chapter 13: Background Document: Reproductive success in eelpout (*Zoarces viviparus*)		Chapter 12	Technical Annex 10
Chapter 14: Background Document: Sediment seawater elutriate and pore-water bioassays with early developmental stages of marine invertebrates		Chapters 7, 8, and 10	Technical Annexes 2-3

Background documents and Technical annexes in Publication 589/2012	**New in 589/2012**	**Chapter updated or replaced in Publication 333/2007**	**Coverage of JAMP Guidelines General Biological Effects**
Chapter 15: Background Document: Sediment seawater elutriate and pore-water bioassays with copepods (Tisbe, Acartia), mysids (Siriella, Praunus), and decapod larvae (Palaemon)		Chapters 7, 8, 9 and 10	Technical Annexes 2-3
Chapter 16: Background Document: Water in vivo bioassays		Chapter 7	Technical Annex 4
Chapter 17: Background Document: Whole sediment bioassays with amphipods (Corophium sp.) and Arenicola marina		Chapter 8	Technical Annex 1
Technical Annex on recommended packages of chemical and biological methods for monitoring on a contaminant basis	Yes		
Technical Annex on sampling and analysis for integrated chemical and biological effects monitoring in fish and shellfish (Revised in 2011)	Yes		
Technical Annex: Assessment criteria for biological effects measurements	Yes		
Technical Annex for integrated chemical and biological monitoring of mussels (*Mytilus* sp.)	Yes		
Technical Annex: Supporting parameters for biological effects measurements in fish and mussels	Yes		
Technical Annex: Protocol for extraction, cleanup, and solvent exchange methods for small-scale bioassays	Yes		
Discussion document on survey design for integrated chemical and biological effects monitoring	Yes		

Background documents and Technical annexes contained in Publication 333/2007 but not in Publication 589/2012	
Scope for growth	Needs to be updated. In progress
Background Document: Fish vitellogenin as a biomarker of exposure to xenoestrogens	Needs to be updated. In progress
Assessment criteria for D-Aminolevulinic acid dehydratase (ALA-D) measured in fish blood	Obsolete
Assessment criteria for Hepatic Metallathionein in fish	Obsolete

Contents

Chapter 1: Acetylcholinesterase assay as a method for assessing neurotoxic effects in aquatic organisms

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 to detect neurotoxic effects of contaminant concentrations occurring in marine waters. AChE activity method 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, while butyrylcholinesterase has the highest specificity for butyrylcholine or propylthiocholine. The inhibition of AChE leads to an accumulation of ACh which, in turn, over-stimulates 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, in the eye and muscle of prawn (Frasco *et al*., 2010). Molluscs in general show low activity (Bocquené *et al*., 1998). In vertebrates, neurotoxic poisoning with hyperactivity, tremors, convulsions and paralysis may finally 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 to 1 $\mu g l^{-1}$; Habig *et al*., 1986).

During the 1990s, there was a resurgence of interest concerning the use of ChEs 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; 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 (Schiedek *et al*., 2006; Kopecka *et al*., 2006, Baršienė *et al*., 2006).

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 the observed depression 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 ChEs in the same tissue having different sensitivities to anti-cholinesterase agents may act as a confounding factor; therefore, prior characterization of the enzymes present is recommended (Garcia *et al*., 2000). AChE activity of juveniles of *Callionymus lyra* in the Atlantic sea and in *Serranus cabrilla* and *Mullus barbatus* in the Mediterranean Sea is higher than that of adults, but no differences were found between male and female in *Limanda limanda* in the Atlantic Ocean (Galgani and Bocquené, 1992).

Different biotic and abiotic factors are known to modulate AChE activity, including trace metals (cadmium copper, mercury, zinc) and variation of natural environmental factors, i.e. seawater temperature and salinity (Pfeifer *et al*., 2005; Leiniö and Lehtonen, 2005; Rank *et al*., 2007). In *Mytilus edulis* and *Macoma balthica* from the northern Baltic Sea, mean values of AChE values vary two-fold depending on season, following

closely changes in temperature (Leiniö and Lehtonen, 2005). Seasonal variability has also been shown as different responses to natural factors in coastal areas compared to offshore sites (Dizer *et al*., 2001; 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. 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 cyanobacteria blooms (e.g. *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*) and *Microcystis aeruginosa* have been also 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) e.g. in *Artemia franciscana* (Gagne and Blaise, 2004).

Moulting rate increases with the 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 (Ozretic and Krajnoviö-Ozretic, 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, the hormesis effects cannot be ignored and represents 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 sensitivity 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, ChEs of some invertebrates have been shown to have some differences in their properties compared to typical forms of vertebrates. For example, ChEs 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.

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 the 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 characterisation of neurotoxic effects of a wide spectrum of organic and inorganic contaminants in the marine environment.

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 to that of chemical analyses, with a detection limit of 100 ngL^{-1} for carbamates and 10 ngL^{-1} for organophosphates (Kirby *et al*., 2000).

Standardisation of the sampling strategy and regular intercalibration exercises on specific organisms sampled in the Atlantic Ocean, Mediterranean 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.

Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC)

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 1). Assessment criteria should be defined on regional basis, using available long-term data.

Therefore, in order to understand and apply the 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 $prot^{-1}$) of the seasonal cycle of the mussel Mytilus edulis studied during three years along the Atlantic coast demonstrated a maximum of amplitude of 30% (Bocquené *et al*., 2004).

Generally, 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% to 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 1). However, differences between sea areas and seasons are obvious, e.g. with activity values in Mytilus spp. varying from 25 to 54 nmol min^{-1} mg $protein^{-1}$.

According to these observations, background assessment criteria (BAC) and environmental assessment criteria (EAC) were proposed using the 10th percentile of data. BACs are estimated from data from reference sites and describe the threshold value for the background level. Environmental Assessment Criteria (EACs) are usually derived from toxicological data and indicate a significant risk to the organism. EACs were calculated by subtracting 30% from the BAC values (Table 1) and represent a significant inhibition of AChE activity. EAC values characterise a sublethal impact. BACs and EACs should be estimated for different geographical regions, and include the effect of differences in water temperature.

Table 1. Assessment of acetylcholinesterase activity after *in vitro* and *in vivo* exposure of biomonitoring organisms in control laboratory conditions and field studies that have utilised common monitoring species collected from reference locations.

Organisms	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 prot-1)	EAC (activity nmol.min-1 mg prot-1)	Ref.
Invertebrates							
Mytilus gallorpovincialis	Gills	Wild mussels Mediterranean Sea in Spain	May–June	15–25	15	10	Campillo-Gonzalez (unpublished results)
Mytilus gallorpovincialis	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 (N.W. Portuguese)	Seasonal cycle		26	19	L.Guilhermino (unpublished results)
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°C	235	165	Burgeot *et al*., 2001
Plathichthys flesus	Muscle	French Atlantic ocean (Ster estuary-Brittany)		15°C	335	235	Evrard *et al*., 2010
Limanda limanda	Muscle	French Atlantic ocean (Seine Bay)		15°C	150	105	Burgeot *et al*., 2001
Mullus barbatus	Brain	Mediterranean Sea SE Spain (Màlaga-Almeria)	October	14 ºC	75	52	Martínez-Gómez, unpublished results
Mullus barbatus	Muscle	Mediterranean Sea (France, Spain, Italy)	*In situ*	18°C	155	109	Burgeot *et al*., 1996, Bocquené, 2004

Future work

Standardised 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 a basis of standardised procedure. 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.

References

Baršienė, J., Lehtonen, K. K., Köhler, A., Broeg, K., Vuorinen, P.J., Lang, T., Pempkowiak, J., Šyvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjärvi, H. and Kopecka, J. 2006. Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipeda-Butinge area (Baltic Sea). Marine Pollution Bulletin 53: 422–436.

Bocquené G., Roig, A. and Fournier D. 1997. Acetylcholinesterases from the common oyster *Crassotrea gigas*. Evidence for the presence of a soluble acetylcholinesterase insensitive to organophosphate and carbamate inhibitors. FEBS Lett 3:261–266.

Bocquené, G. and Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences. No 22.12p. ICES Copenhagen DK.

Bocquené, G., P. 2004. Biological effects of environmental pollution in aquatic ecosystem. BEEP WEB. NOE Global change and ecosystem 6.3. (Personal communication).

Bocquené, G., Chantereau, S., Raffin, B., Minier, C., Clérendeau, C., Leskowicz, A., Beausir, E., Burgeot, T. and Ménard, D. 2004. The monitoring of the biological effects of the ERIKA oil spill on the blue mussel (*Mytilus edulis*). Aquatic Living Resources. 17.309–316.

Bodin, N., Burgeot, T., Stanisière, J.Y., Bocquené, G., Ménard, D., Minier, C., Boutet, I., Amat, A. Cherel, Y. and Budzinski, H. 2004. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the Northwest Mediterranean Sea. Comparative Biochemistry and Physiology. 138:411–427.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B. and Depledge, M.H. 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol, 66. 267–278.

Burgeot, T., G. Bocquené, C. Porte, A., Pfhol Leszkowicz, Santella, R.M., Raoux, C., Dimeet, J. and Galgani, F. 1996. Bioindicators of pollutant exposure in the northwestern part of the Mediterranean Sea. Mar. Ecol. Prog. Ser.131: 125–141.

Burgeot, T., Bocquené, G., His, E., Vincent, F., Geffard, O., Beiras, R., Goraguer, H. and Galgani, F. 2001. Procedures for cholinesterase determination in fish and mussel. Technical annex. In: Biomarkers in marine organisms; a practical approach. Edited by Garrigues, P., Barth, H., Walker, C.H. and Narbonne, J.F. Eds Elsevier.487p.

Burgeot, T., Faucet, J., Ménard, D., Grosjean, P. and Bocquené, G. 2006. Variations of benzo(a)pyrene hydroxylase and cholinesterase activities in mussels caged in the North Sea (German Bight and Statfjord). Special Issue. Environmental Toxicology and chemistry. 171–185.

Busby, D.G., White, L.M., Pearce, P.A. and Mineau, P. 1989. Fenitrothion effects on forest songbirds: a critical new look. In: Environmental effects of fenitrothion use in forestry, Ed.: Ernst, W.R. *et al* Environment Canada Publication, no EN40370/1989E, p.43–108.

Coppage, D.L., Braidech, T.E. 1976. River pollution by antilcholinesterase agents. Water Research, 10: 19–24.

Cunha, I., Garcia, L.M., Guilhermino, L. 2005. Sea-urchin (*Paracentrotus lividus*) glutathione S-transferases and cholinesterase activities as biomarkers of environmental contamination. Journal of Environmental Monitoring 7: 288–294.

Cunha, I., Mangas-Ramirez, E., Guilhermino, L. 2007. Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (*Monodonta lineata and Nucella lapillus*). Comparative Biochemistry and Physiology Part C: Toxicology and Pharmacology 145: 648–657.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M. and Dimitriadis, V.K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Marine Environmental Research 56, 443–470.

Day, K.E. and Scott, I.M. 1990. Use of acetylcholinesterase activity to detect sublethal toxicity in stream invertebrates exposed to low concentrations of organophosphate insecticides. Aquatic Toxicology, 18: 101–114.

Diamantino, T.C., Almeida, E., Soares, A.M.V.M., Guilhermino, L. 2003. Characterization of cholinesterases from *Daphnia magna* Straus and their inhibition by zinc. Bulletin of Environmental Contamination and Toxicology 71: 219–225.

Dizer, H., da Silva de Assis, H.C. and Hansen, P.-D. 2001. Cholinesterase activity as a bioindicator for monitoring marine pollution in the Baltic Sea and the Mediterranean Sea. pp331–342. In: Biomarkers in marine organisms; a practical approach. Edited by Ph. Garrigues, H. Barth, C.H. Walker and J.F. Narbonne. Eds Elsevier.491p.

Evrard, E., Devaux, A., Bony, S., Burgeot, T., Riso, R., Budzinski, H., Ledu, M., Quiniou, L., Laroche, J. 2010. Response of the European flounder *Platychthys flesus* to the chemical stress in estuaries: load of contaminants, gene expression, cellular impact and growth rate. Biomarkers. 1–17.

Frasco, M.F., Fournier, D., Carvalho, F. and Guilhermon, L. 2005. Do metal inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers, 10, 360–375.

Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L. 2006. Cholinesterase from the common prawn (*Palaemon serratus*) eyes: catalitic properties and sensitivity to organophosphate and carbamate compounds. Aquatic Toxicology 77: 412–421.

Frasco, M.F., Erzen, I., Stojan, J., Guilhermino, L. 2010. Localization and properties of cholinesterases in the common prawn (Palaemon serratus): a kinetic-histochemical study. Biological Bulletin 218: 1–5.

Gagne, F. and Blaise, C. 2004. Shell protein characteristics and vitellogenin-like proteins in brine shrimp *Artemia fransciana* exposed to municipal effluent and 20-hydroxyecdsone. Comp. Biochem. Physiol. Part C Pharmacol.Toxicol 138. 515–522.

Galgani, F., Bocquené, G. and Cadiou, Y. 1992. Evidence of variation in cholinesterase activity in fish along a pollution gradient in the North sea. Mar. ecol. Prog. Ser. 91: 1–3).

Galgani, F., Bocquené, G. and Burgeot, T. 1996. Acetylcholinesterase and ethoxyresorufin-o-deethylase in the surgeonfish *Acanthurus bahianus* around Martinique Island (French West Indies). Biomarkers 1. 1–3.

Garcia, L.M., Castro, B., Ribeiro, R., Guilhermino, L. 2000. Characterization of cholinesterase from guppy (*Poecilia reticulata*) muscle and its in vitro inhibition by environmental contaminants. Biomarkers 5: 274-284.

Guilhermino, L., Barros, P., Silva, M.C., Soares, A.M.V.M. 1998. Should the use of inhibition of cholinesterases as a specific biomarker for organophosphate and carbamate pesticides be questioned? Biomarkers 3:157–163.

Habig, C., Di Guilio, R.T., Nomeir, A.A. and Abou Donia, A.M.B. 1986. Comparative toxicity, cholinergic effects and tissue levels of S,S,S-tri-nButyl Phosphoritrithioate 'DEF) to Channel catfish (*Ictarus punctatus*) and blue crab (*Callinectes sapidus*). Aquatic Toxicology, 9:193–206.

Hansson, T., Schiedek, D., Lehtonen, K.K., Vuorinen, P.J., Liewenborg, B., Noaksson, E., Tjärnlund, U., Hanson, M. and Balk, L. 2006. Biochemical biomarkers in adult female perch (*Perca fluviatilis*) in a chronically polluted gradient in the Stockholm recipient (Sweden). Marine Pollution Bulletin 53: 451–468.

Hoguet, J. and Key, P.B. 2007. Activities of biomarkers in multiple life stages of the model crustacean, *Palaemonetes pugio*. Journal of experimental marine biology and ecology. 235–244.

Kankaanpää, H., Leiniö, S., Olin, M., Sjövall, O., Meriluoto, J. and Lehtonen, K. K. 2007. Accumulation and depuration of cyanobacterial toxin nodularin and biomarker responses in the mussel *Mytilus edulis*. Chemosphere 68: 1210–1217.

Kefford, B.J., Zalizniak, L, Warne, M.J., Nugegoda, D. 2008. Is the integration of hormesis and essentiality into ecotoxicology now opening Pandora's box? Environmental pollution 151:516–523.

Kirby, M.F., Morris, S., Hurst, M. Kurby, S.J., Neall, P., Tylor, T. and Fagg, A. 2000. The use of cholinesterase activity in flounder (*Platichthys flesus*) muscle tissue as a biomarker of neurotoxic contamination in UK estuaries. Mar. Pollut. Bull. 9:780–791.

Kopecka, J., Lehtonen, K.K., Baršienė, J., Broeg, K., Vuorinen, P.J., Gercken, J., Balk, L. and Pempkowiak, J. 2006. Measurements of biomarker levels in flounder (*Platichthys flesus*) and blue mussel (*Mytilus trossulus*) from the Gulf of Gdańsk (southern Baltic). Marine Pollution Bulletin 53: 406–421.

Lehtonen, K.K., Kankaanpää, H., Leiniö, S., Sipiä, V.O., Pflugmacher, S., and Sandberg-Kilpi, E. 2003. Accumulation of nodularin-like compounds from the cyanobacterium *Nodularia spumigena* and changes in acetylcholinesterase activity in the clam *Macoma balthica* during short-term laboratory exposure. Aquatic Toxicology 64: 461–476.

Lehtonen, K.K. and Leiniö, S. 2003. Effects of exposure to copper and malathion on metallothionein levels and acetylcholinesterase activity of the mussel *Mytilus edulis* and the clam *Macoma balthica* from the northern Baltic Sea. Bulletin of Environmental Contamination and Toxicology 71: 489–496.

Leiniö, S. and Lehtonen, K.K. 2005. Seasonal variability in biomarkers in the bivalves Mytilus edulis and Macoma balthica from the northern Baltic Sea. Comparative Biochemistry and Physiology C 140: 408–421.

Ozretið, B. and Krajnovið-Ozretið, M. 1992. Esterase heterogeneity in mussel *Mytilus galloprovincialis*: effects of organophosphate and carbamate pesticides in vitro. Comp Biochem Physiol 103:221–225.

Payne, J.F., Mathieu, A., Melvin, W. and Fancey, L.L. 1996. Acetylcholinesterase, an old biomarker with a new future? Field trials in association with two urban rivers and a paper mill in Newfoundland. Mar. Pollut. Bull. 32:225–231.

Pfeifer, S., Schiedek, D. and Dippner, J.W. 2005. Effect of temperature and salinity on acetylcholinesterase activity, a common pollution biomarker, in *Mytilus* sp. from the south-western Baltic Sea. J. exp. mar. biol. ecol. 320: 93–103.

Printes, L.B. and Callaghan, A. 2004. A comparative study on the relationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. Environ. Toxicol.Chem.23, 1241–1247.

Rank, J., Lehtonen, K.K., Strand, J. and Laursen, M. 2007. DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (Mytilus edulis) in areas close to coastal chemical dumping sites in Denmark. Aquatic Toxicology 84: 50–61.

Rodrigues, S.R., Caldeira, C., Castro, B.B., Gonçalvez, F., Nunes, B. and Antunes, S.C. 2011. Cholinesterase (ChE) inhibition in pumpkinseed (*Lepomis gibbosus*) as environmental biomarker: ChE characterization and potential neurotoxic effects of xenobiotics. Pesticide Biochemistry and Physiology. 99 : 181–188.

Schiedek, D., Broeg, K., Baršienė, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Schneider, R., Vuontisjärvi, H., Vuorinen, P.J., Koehler, A. and Balk, L. 2006. Biomarker responses and indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the western Baltic Sea. Marine Pollution Bulletin. 53: 387–405.

Signa, G., Cartes, J.E., Sole, M.., Serrano, A. and Sanchez, F. 2008. Trophic ecology of the swimming crab *Polybius henslowii* Leach, 1820 in Galician and Cantabrian Seas: Influences of natural variability and the Prestige oil spill. Continental Shelf Research. 2659–2667.

Tim-Tim, A.L., Morgado, F., Moreira, S., Rangel, R., Nogueira, A.J., Soares, A.M., Guilhermino, L. 2009. Cholinesterase and glutathione S-transferase activities of three mollusk species from the NW Portuguese coast in relation to the 'Prestige' oil spill. Chemosphere 77: 1465–1475.

Varó, I., Navarro, J.C., Amat, F., Guilhermino, L. 2002. Characterisation of cholinesterases and evaluation of the inhibitory potential of chlorpyrifos and dichlorvos to *Artemia salina* and *Artemia parthenogenetica*. Chemosphere 48: 563–569.

Vieira, L.R., Gravato, C., Soares, A.M.V.M., Morgado, F., Guilhermino, L. 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: linking biomarkers to behaviour. Chemosphere 76: 1416–1427.

Zinkl, J.G., Shea, P.J., Nakampoto, R.J. and Callman, J. 1987. Technical and biological considerations for the analysis of brain cholinesterase of rainbow trout. Trans.Am. Soc. 116: 570–573.

Zinkl, J.G., Lockhart, W.L., Kenny, S.A. and Ward, F.J. 1991. The effects of cholinesterase inhibiting insecticides on fish. In: Mineau P (ed) Cholinesterase Inhibiting Insecticides. Elsevier, Amsterdam, pp. 233–254.

Chapter 2: Comet assay as a method for assessing DNA damage in aquatic organisms

Background

The analysis of modified or damaged DNA has been shown to be a highly suitable 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 Férard, 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:

(1) genotoxic damage can be detected in most eukaryotic cell types at the single cell level;

(2) only a small number of cells are required;

(3) it is a rapid and sensitive technique;

(4) due to the nature of DNA strand break formation it provides an early warning response of genotoxic exposure;

(5) sites of oxidative damage can be identified using enzymatic pre-treatment.

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). While 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.

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 due to 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, with the potential of introducing damage through enzymatic or mechanical processes. Studies have also demonstrated that different cell types responded 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; Pandey *et al*., 2006; Kim and Hyun, 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 MNNG exposure, while liver and digestive gland were more sensitive to B(a)P, suggesting that uptake routes and bioaccumulation mechanisms need to be taken into account when designing experiment systems (Kim and Hyun, 2006). Mammalian studies have demonstrated that certain tissue types may have higher background levels of DNA damage due to 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 (Frenzilli *et al*., 1999; Moretti *et al*., 1998). 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 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 Férard, 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 type commonly used in environmental studies. The production of standard protocols or the initiation of inter-laboratory ring testing workshops focused on aquatic species is 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 (Hylland *et al*., in prep), which will make the assay more directly applicable for monitoring purposes.

Ecological relevance

Marine invertebrates (bivalves)

Marine invertebrates have been widely used as sentinel species in environmental monitoring programs. This is mainly due to, their ability to bio-accumulate 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*., 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 (Hartl *et al*., 2004). 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; Laffon *et al*., 2006; Taban *et al*., 2004). 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*) has 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 (Rank *et al*., 2007; Regoli *et al*., 2004; Nigro *et al*., 2006). 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 under taken with the comet assay to assess the time course variations in DNA damage following field transplantation experiments (Rank *et al*., 2007; Regoli *et al*., 2004). It was observed that within the first seven days following transplantation the level of DNA damage can fluctuate, which is likely to be caused by manipulation disturbance, then after two weeks the level reaches a plateau. Such data suggests that transplantation experiments lasting less than two weeks 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, impacted 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 the 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 (Halldorsson *et al*., 2004). While the study supports the use of DNA strand breaks as a measure of environmental pollution it also highlights the high levels of intra site 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.

Marine vertebrates (fish)

There are a limited number of comet assay studies utilising marine fish species in comparison to those using freshwater species (for detailed review see Mitchelmore and Chipman, 1998; Jha, 2008; Fenzilli *et al*., 2009). This is mainly due to the logistical problems associated with collecting fish at sea (*e.g.* need for a research vessels) 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 those studies undertaken have mainly focused on flatfish and bottom-feeding species, which due to their close association with sediment bound contaminants are widely used in marine monitoring programmes (Feist *et al*., 2004; Oslo and Paris Commissions, 1998). *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, the environmental effects of the known mutagen and potentia. Styrene has not previously been considered to be harmful to marine fauna due to its high volatility l carcinogen styrene has been studied in the mussel (*M. edulis*) and fish (*Symphodus mellops*) (Mamaca *et al*., 2005) and low capacity to bio-accumulate. However, it was shown to cause a statistically significant increase in DNA damage in blood cells, probably due to 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 Goteborg harbour, Sweden. The comet assay was deployed along site a battery of other bioassays and elevated levels of DNA damage were correlated to 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; Akcha *et al*., 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; Akcha *et al*., 2004).

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 :

1) cell isolation processes (if required);
2) cryopreservation processes;
3) concurrent measures of cytotoxicity;
4) Image analysis and scoring method.

Currently data can be reported in a number of formats. % 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 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.

Background responses and assessment criteria

It is recognised 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 1 summaries a number of studies that have utilised commonly deployed bioindicator species collected from reference locations (as supported by chemical and biomarker analyses) or kept under control conditions in the laboratory. While 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 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	Ref.
Invertebrates						
M. edulis	Haemocytes	MMS	0-4 days	Tail Moment	2.08 ± 3.43 2.96 ± 4.60	(Rank *et al*., 2007)
M. edulis	Haemocytes	Tritiated water	96 hrs	% DNA Tail	<10	(Jha *et al*., 2005)
M. edulis	Haemocytes	TBT	7 days	% 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 days 7 days 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_20_2, BaP	1hr	% 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)	<10	(Woo *et al*., 2006)
Z. viviparus	Erythrocytes	Field	*In situ*	% DNA Tail	<15	(Frenzilli et al., 2004)

*Mean square root of percent tail DNA measured.

In addition to the above, there was a recent study as part of ICON in which dab (*Limanda limanda*) were collected from the North Sea and in Icelandic waters (Skei *et al*., in prep). 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 (Sanni, unpubl). The water was supplied continuously from a non-polluted source at 78 meters depth of 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 found 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, while 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 is not sufficient data to provide an 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 an 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 QA and intercalibration exercises (will be initiated by WGBEC members) and further evaluation of the suggested assessment criteria.

References

Akcha, F., Leday, G. and Pfohl-Leszkowicz, A. 2003. Potential value of the comet assay and DNA adduct measurement in dab (*Limanda limanda*) for assessment of in situ exposure to genotoxic compounds, Mutat. Res. 534, 21-32.

Akcha, F., Vincent, H.F. and Pfohl-Leszkowicz, A. 2004. Measurement of DNA adducts and strand breaks in dab (*Limanda limanda*) collected in the field: effects of biotic (age, sex) and abiotic (sampling site and period) factors on the extent of DNA damage, Mutat. Res. 552, 197-207.

Baussant, T., Bechmann, R.K., Taban, I.C., Larsen, B.K., Tandberg, A.H., Bjørnstad, A., Torgrimsen, S., Nævdal, A., Øysæd, K.B., Jonsson, G. and Sanni, S. 2009. Enzymatic and cellular responses in relation to body burden of PAHs in bivalve molluscs: A case study with chronic levels of North Sea and Barents Sea dispersed oil. Marine Poll Bull, 58, 1796-1807

Bayne, B.L. 1976. Watch on mussels. Mar. Pollut. Bull., 7(12), 217-128.

Bignell, J.P., Dodge, M.J., Feist, S.W., Lyons, B.P., Martin, P.D., Taylor, N.G.H., Stone, D., Travalent, L., Stentiford, G.D. 2008. Mussel histopathology: effects of season, disease and species. Aquatic Biology, 2, 1-15.

Burlinson, B., Tice, R.R., Speit, G., Agurell, E., Brendler-Schwaab, S.Y., Collins, A.R., Escobar, P., Honma, M., Kumaravel, T.S., Nakajima, M., Sasaki, Y.F., Thybaud, V., Uno, Y., Vasquez, M. and Hartmann, A., 2007., Fourth International Workgroup on Genotoxicity testing: results of the *in vivo* Comet assay workgroup, Mutat. Res. 627. 31-35.

Canty, M.N., Hutchinson, T.H., Brown, R.J., Jones, M.B., Jha, A.N. 2009. Linking genotoxic responses with cytotoxic and behavioural or physiological consequences: Differential sensitivity of echinoderms (*Asterias rubens*) and marine molluscs (*Mytilus edulis*). Aquat. Toxicol., 94 (1), pp. 68-76.

Cotelle, S. and. Férard, J.F. 1999. Comet assay in genetic ecotoxicology: a review, Environ. Mol. Mutagen. 34, 246-255.

Emmanouil, C., Smart, D.J., Hodges, N.J., Chipman, J.K. 2006. Oxidative damage produced by Cr(VI) and repair in mussel (*Mytilus edulis L*.) gill. Mar. Environ. Res. 62 (SUPPL. 1), pp. S292-S296.

Feist, S.W., Lang, T., Stentiford, G.D., Köhler, A. 2004. Biological effects of contaminants: use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES Techniques in Marine Environmental Sciences 38. 42 pp.

Fenzilli, G., Nigro, M., Lyons, B.P. 2009. The Comet assay for evaluation of genotoxic impact in aquatic environments. Mutat Res. 681, 80-92.

Frenzilli, G., Scarcelli, V., Taddei, F. and Nigro, M. 1999. Adaption of SCGE as a candidate for monitoring marine ecosystems, Neoplasma 46, 6-7.

Frenzilli, G., Scarcelli, V., Del Barga, I., Nigro, M., Forlin, L., Bolognesi, C. and Sturve, J. 2004. DNA damage in eelpout (*Zoarces viviparus*) from Goteborg harbour, Mutat. Res. 552, 187-195.

Halldorsson, H.P., Ericson, G. and Svavarsson, J. 2004. DNA strand breakage in mussels (*Mytilus edulis L*.) deployed in intertidal and subtidal zone in Reykjavik harbour, Mar. Environ. Res. 58, 763-767.

Hagger, J.A., Depledge, M.H., Galloway, T.S. 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. Mar. Poll. Bull. 51 (8-12), pp. 811-816.

Hartl, M.G., Coughlan, B.M., Sheehan, D., Mothersill, C., van Pelt, F.N., O'Reilly, S.J., Heffron, J.J., O'Halloran, J. and O'Brien, N.M.. 2004. Implications of seasonal priming and reproductive activity on the interpretation of Comet assay data derived from the clam, *Tapes semidecussatus* Reeves 1864, exposed to contaminated sediments, Mar. Environ. Res. 57, 295-310.

Hartl, M.G.J., Kilemade, M., Sheehan, D., Mothersill, C., O'Halloran, J., O'Brien, N.M. and van Pelt, F.N.A.M. 2007. Hepatic biomarkers of sediment-associated pollution in juvenile turbot, *Scophthalmus maximus* L, Mar. Environ. Res. 64, 191-208.

Hines, A., Oladirana, G.S., Bignell, J.P., Stentiford, G.S., Viant, M.R. 2007. Direct sampling of organisms from the field and knowledge of their phenotype: key recommendations for environmental metabolomics, Environ. Sci. Tech, 41, 3375-3381.

Huang, D., Zhang, Y., Wang, Y., Xie, Z. and Ji, W. 2007. Assessment of the genotoxicity in toad *Bufo raddei* exposed to petrochemical contaminants in Lanzhou Region, China, Mutat. Res. 629, 81-88.

Jha, A.N., Dogra, Y., Turner, A., Millward, G.E. 2005. Impact of low doses of tritium on the marine mussel, *Mytilus edulis*: Genotoxic effects and tissue-specific bioconcentration. Mut. Res. Genet. Toxicol. Environ. Mutagen 586 (1), pp. 47-57.

Jha, A.N. 2008. Ecotoxicological applications and significance of the comet assay, *Mutagenesis* 23 (3), 207-221.

Kim, I.Y. and Hyun, C.K. 2006. Comparative evaluation of the alkaline comet assay with the micronucleus test for genotoxicity monitoring using aquatic organisms, Ecotoxicol. Environ. Saf. 64, 288-297.

Laffon, B., Rabade, T., Pasaro, E. and Mendez, J. 2006. Monitoring of the impact of Prestige oil spill *on Mytilus galloprovincialis* from Galician coast, Environ. Int. 32, 342-348.

Lee, R.F. and Steinert, S. 2003. Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals, Mutat. Res. 544, 43-64.

Lemière, S., Cossu-Leguille, C., Bispo, A., Jourdain, M.J., Lanhers, M.C., Burnel, D. and Vasseur, P. 2005. DNA damage measured by the single-cell gel electrophoresis (Comet) assay in mammals fed with mussels contaminated by the "Erika" oil-spill, Mutat. Res. 581, 11-21.

Lyons, B.P., Stentiford, G.D., Bignell, J., Goodsir, F., Sivyer, D.B., Devlin, M.J., Lowe, D., Beesley, A., Pascoe, C.K, Moore, M.N. and Garnacho, E. 2006. A biological effects monitoring survey of Cardigan Bay using flatfish histopathology, cellular biomarkers and sediment bioassays: findings of the Prince Madog Prize 2003, Mar. Environ. Res. 62, S342-S346.

Mamaca, E., Bechmann, R.K., Torgrimsen, S., Aas, E., Bjornstad, A., Baussant, T. and Floch, S.L. 2005. The neutral red lysosomal retention assay and Comet assay on haemolymph cells from mussels (*Mytilus edulis*) and fish (*Symphodus melops*) exposed to styrene, Aquat. Toxicol. 75, 191-201.

Mitchelmore, C.L., Birmelin, C., Livingstone, D.R., Chipman, J.K. 1998. Detection of DNA Strand Breaks in Isolated Mussel (*Mytilus edulis L*.) Digestive Gland Cells Using the "Comet" Assay. Ecotox and Environ. Saf. 41, 51D58 (1998).

Mitchelmore, C.L. and Chipman, J.K. 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring, Mutat. Res. 399, 135-147.

Moretti, M., Villarini, M., Scassellati-Sforzolini, G., Santroni, A.M., Fedeli, D. and Falcioni, G. 1998. Extent of DNA damage in density-separated trout erythrocytes assessed by the "comet" assay, Mutat. Res. 397, 353-360.

Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F. and Frenzilli, G. 2006. Cellular biomarkers for monitoring estuarine environments: transplanted versus native mussels, Aquat. Toxicol. 77, 339-347.

Oslo and Paris Commissions, 1998. JAMP guidelines for general biological effects monitoring. Joint Assessment and Monitoring Programme. 38 pp.

Pandey, S., Nagpure, N.S., Kumar, R., Sharma, S., Srivastava, S.K. and Verma, M.S. 2006. Genotoxicity evaluation of acute doses of endosulfan to freshwater teleost *Channa punctatus* (Bloch) by alkaline single-cell gel electrophoresis, Ecotoxicol. Environ. Saf. 65, 56-61.

Perez-Cadahia, B., Laffon, B., Pasaro, E. and Mendez, J. 2004. Evaluation of PAH bioaccumulation and DNA damage in mussels (*Mytilus galloprovincialis*) exposed to spilled Prestige crude oil, Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 138, 453-460.

Rank, J., Jensen, K. and Jespersen, P.H. 2005. Monitoring DNA damage in indigenous blue mussels (*Mytilus edulis*) sampled from coastal sites in Denmark, Mutat. Res./Genet. Toxicol. Environ. Mutagen. 585, 33-42.

Rank, J., Lehtonen, K.K., Strand, J. and Laursen, M. 2007. DNA damage, acetylcholinesterase activity and lysosomal stability in native and transplanted mussels (*Mytilus edulis*) in areas close to coastal chemical dumping sites in Denmark, Aquat. Toxicol. 84, 50-61.

Rank, J. 2009. Intersex in Littorina littorea and DNA damage in *Mytilus edulis* as indicators of harbour pollution Ecotox. Environ. Saf., 72 (4), pp. 1271-1277.

Regoli, F., Winston, G.W., Gorbi, S., Frenzilli, G., Nigro, M., Corsi, I. and Focardi, S. 2003. Integrating enzymatic responses to organic chemical exposure with total oxyradical absorbing capacity and DNA damage in the European eel *Anguilla anguilla*: toward development of a more holistic biomarker assessment, Environ. Toxicol. Chem. 22, 2120-2129.

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D. and Nigro, M. 2004. Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment, Aquat. Toxicol. 68, 167-178.

Salazar, M.H. and Salazar, S.M. 1995. *In situ* bioassays using transplanted mussels: I. Estimating chemical exposure and bioeffects with bioaccumulation and growth. In Hughes JS, Biddinger GR, Mones E, Eds. Environmental Toxicology and Risk Assessment . Third Volume. Philadelphia: American Society for Testing and Materials STP, 2118, 216-241.

Seed, R. 1976. Ecology. In: B.L. Bayne, Marine Mussels, their ecology and physiology, International Biological Programme (13-65), Cambridge University Press 13-65.

Singh, N.P., Danner, D.B., Tice, R.R., McCoy, M.T., Collins, G.D. and Schneider, E.L. 1989. Abundant alkali-sensitive sites in DNA of human and mouse sperm, Exp. Cell. Res. 184, 461-470.

Siu, W.H., Cao, J., Jack, R.W., Wu, R.S., Richardson, B.J., Xu, L. and Lam, P.K. 2004. Application of the comet and micronucleus assays to the detection of B[*a*]P genotoxicity in haemocytes of the green-lipped mussel (*Perna viridis*), Aquat. Toxicol. 66, 381-392.

Taban, I.C., Bechmann, R.K., Torgrimsen, S., Baussant, T. and Sanni, S. 2004. Detection of DNA damage in mussels and sea urchins exposed to crude oil using comet assay, Mar. Environ. Res. 58, 701-705.

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C and Sasaki, Y.F. 2000. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing, Environ. Mol. Mutagen. 35, 206-221.

Wirzinger, G., Weltje, L., Gercken, J. and Sordyl, H. 2007. Genotoxic damage in field-collected three-spined sticklebacks (*Gasterosteus aculeatus L.*): a suitable biomonitoring tool? Mutat. Res. 628, 19-30.

Woo, S., Kim, S., Yum, S., Yim, U.H., Lee, T.K. 2006. Comet assay for the detection of genotoxicity in blood cells of flounder (*Paralichthys olivaceus*) exposed to sediments and polycyclic aromatic hydrocarbons Mar. Poll. Bull. 52 (12), pp. 1768-1775.

Chapter 3: Cytochrome P4501A activity (EROD)

Introduction

The cytochrome P450 1A family of enzymes are responsible for the primary metabolism of planar polyaromatic hydrocarbons and PCBs and activate 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 Meyer (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 Ah (aryl hydrocarbon)- 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) which has now gained widespread use (see *e.g.* Förlin *et al*., 1990; George *et al*., 1995; Goksøyr *et al*., 1987; Whyte *et al*., 2000) and standardised by ring testing (BEQUALM).

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 10-fold 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 categorised 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).

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*., 1995; Lange *et al.*, 1999). Seasonal cycles in EROD induction have been observed for *i.e.* rainbow trout (Förlin & Haux 1990), flounder Hylland *et al*., 1996), plaice and salmon (Larsen *et al*., 1992), most likely due to both to changes in 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 10-fold EROD induction after contaminant exposure (Whyte *et al.,* 2000). These are, however, mostly freshwater species.

CYP1A expression is suppressed in spawning females due to interference of 17β-estradiol (E_2) (or xenoestrogen) with transcription of the gene. This may also lead to an under estimation 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 recrudescene. Therefore interference of environmental estrogens on CYP1A induction can be assessed.

Dietary factors can be potentially important for the induction of CYP1A. Firstly, of course, AhR ligands can be presented to the organism through the food. Secondly, proper nutrition is a prerequisite for enzyme systems to function properly. Hylland *et al*. (1996) reported an elimination of EROD response (*i.e.* to control levels) in BaP-treated flounder deprived of food for one month.

Background responses

Baseline levels of EROD in seven marine species have been estimated from results derived from the joint ICES/OSPAR WKIMON III meeting (2007) and recent data submitted to ICES database (Table 1). The fish were from sites which Contracting Parties consider being reference stations (*i.e*. no known local sources of contamination or those areas which were not considered unequivocally as reference sites but as those less influenced from human and industrial activity. The data sets from which these values have been derived are described in Table 2. Further information on the baseline levels and dose response of EROD activity in experimental systems and field studies is given in Tables 3 and 4.

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. As many factors are known to influence EROD activity (see above) and 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 2 will also allow data to be assessed against the appropriate assessment criteria for fish species, gender, size, sampling season and bottom water temperature.

Quality assurance

Cytochrome P4501A is possibly the most widely used biomarker. There have been three international intercalibrations for the method, both within BEQUALM. The intercalibrations have pinpointed variability relating to most steps in the analytical process, excepting 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.

Acknowledgement

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Relevant literature (marine and freshwater fish)

Aas, E., Baussant, T., Balk, L., Liewenborg, B., and Andersen, O.K. 2000. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquatic Toxicology, 51: 241-258.

Bernhoft, A., Hektoen, H., Skaare, J. U., and Ingebrigtsen, K. 1994. Tissue distribution and effects on Hepatic Xenobiotic-Metabolizing enzymes of 2,3,3',4,4'-Pentachlorobiphenyl (Pcb-105) In Cod (*Gadus-Morhua*) and Rainbow Trout (Oncorhynchus-Mykiss). Environmental Pollution: 85, 351-359.

Beyer, J., Sandvik, M., Hylland, K., Fjeld, E., Egaas, E., Aas, E., Skare, J.U., and Goksoyr, A. 1996. Contaminant accumulation and biomarker responses in flounder (*Platichthys flesus* L) and Atlantic cod (*Gadus morhua* L) exposed by caging to polluted sediments in Sorfjorden, Norway. Aquatic Toxicology, 36: 75-98.

Burke, M.D., and Mayer, R.T. 1974. Ethoxyresorufin: Direct fluorimetric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylchoalanthrene. Drug metabolism and Disposition, 2: 583-588.

Cooreman, K., Roose, P. and Vyncke, W. 1993. EROD monitoring in dab from the Belgian continental shelf. ICES CM 1993/E:14.

Förlin, L., and Haux, C. 1990. Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. J. Endocrinol., 124: 207-213

Förlin, L., Lemaire, P., and Livingstone, D.R. 1995. Comparative studies of hepatic xenobiotic metabolizing and antioxidant enzymes in different fish species. Marine Environmental Research, 39: 201- 204.

George, S.G., Christiansen, J.S., Killie, B., and Wright, J. 1995. Dietary crude oil exposure during sexual maturation induces hepatic mixed function oxygenase (Cyp1a) activity at very low environmental temperatures in Polar Cod *Boreogadus-Saida*. Marine Ecology Progress Series, 122: 307-312.

George, S.G., Wright, J., and Conroy, J. (1995). Temporal Studies of the impact of the Braer Oilspill on inshore feral fish from Shetland, Scotland. Archives of environmental contamination and Toxicology, 29: 530-534. Goksoyr, A., Andersson, T., Buhler, D.R., Stegeman, J.J., Williams, D.E., and Förlin, L. 1991. Immunochemical cross-reactivity of Beta-Naphthoflavone-Inducible Cytochrome P450 (P450ia) in liver microsomes from different fish species and rat. Fish Physiology And Biochemistry, 9: 1-13.

Goksoyr, A., Andersson, T., Hansson, T., Klungsoyr, J., Zhang, Y., and Förlin, L. 1987. Species characteristics of the hepatic xenobiotic and steroid biotransformation systems of 2 teleost fish, Atlantic Cod (*Gadus-Morhua*) and Rainbow Trout (*Salmo-Gairdneri*). Toxicology and Applied Pharmacology, 89: 347-360.

Goksoyr, A., Beyer, J., Husoy, A.M., Larsen, H.E., Westrheim, K., Wilhelmsen, S., and Klungsoyr, J. 1994. Accumulation and effects of aromatic and chlorinated hydrocarbons in juvenile Atlantic Cod (*Gadus- Morhua*) caged in a polluted fjord (Sorfjorden, Norway). Aquatic Toxicology, 29: 21-35.

Hasselberg, L., Meier, S., Svardal, A., Hegelund, T., and Celander, M.C. 2004. Effects of alkylphenols on CYP1A and CYP3A expression in first spawning Atlantic cod (*Gadus morhua*). Aquatic Toxicology, 67: 303-313.

Hektoen, H., Bernhoft, A., Ingebrigtsen, K., Skaare, J. U., and Goksoyr, A. 1994. Response of hepatic xenobiotic metabolizing enzymes in Rainbow Trout (Oncorhynchus-Mykiss) and Cod (*Gadus-Morhua*) to 2,3,7,8- Tetrachlorodibenzo-P-Dioxin (2,3,7,8-Tcdd). Aquatic Toxicology, 28: 97-106.

Hylland, K., Ruus, A., Børseth, J.F., Bechmann, R., Barsiene, J., Grung, M., Tollefsen, K., Myhre, L.P. 2006. Biomarkers in monitoring - a review. NIVA-report 5205. 106 pp.

Hylland, K., Sandvik, M., Skåre, J.U., Beyer, J., Egaas E, Goksøyr, A. (1996) Biomarkers in flounder (*Platichthys flesus*): an evaluation of their use in pollution monitoring. Mar. Environ. Res. 42:223-227.

Kloeppersams, P.J., and Benton, E., 1994. Exposure of fish to biologically treated bleached-kraft effluent. 2. Induction of Hepatic Cytochrome-P4501a in Mountain Whitefish (Prosopium-Williamsoni) and other species. Environmental Toxicology and Chemistry, 13: 1483-1496.

Lange, U., Goksoyr, A., Siebers, D., Karbe, L. 1999. Cytochrome P450 1A-dependent enzyme activities in the liver of dab (*Limanda limanda*): kinetics, seasonal changes and detection limits. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology, 123: 361-371.

Larsen, H.E., Celander, M. and Goksøyr, A. 1992. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. Fish Physiol. Biochem., 10: 291-301.

Lindesjoo, E., Husoy, A.M., Petterson, I., and Förlin, L. 1996. Histopathological and immunohistochemical studies in roundnose grenadier (*Coryphaenoides rupestris*) in the Skagerrak, North Sea. Marine Environmental Research, 42: 229-233.

Livingstone, D.R., Lemaire, P., Matthews, A., Peters, L.D., Porte, C., Fitzpatrick, P.J., Förlin, L., Nasci, C., Fossato, V., Wootton, N., and Goldfarb, P. 1995. Assessment of the impact of organic pollutants on Goby (*Zosterisessor Ophiocephalus*) and Mussel (*Mytilus-Galloprovincialis*) from the Venice Lagoon,

Italy - Biochemical-Studies. Marine Environmental Research, 39: 235- 240.

Martínez-Gómez, C., Fernández, B., Valdés, J., Campillo, J.A., Benedicto, J., Sánchez, F., and Vethaak, A.D. 2009. Evaluation of three-year monitoring with biomarkers in fish following the Prestige oil spill (N Spain). Chemosphere, 74(5): 613-620.

McDonald, S.J., Willett, K.L., Thomsen, J., Beatty, K.B., Connor, K., Narasimhan, T.R., Erickson, C.M., and Safe, S.H. 1996. Sublethal detoxification responses to contaminant exposure associated with offshore production platforms. Canadian Journal of Fisheries and Aquatic Sciences, 53: 2606-2617.

Michel, X., Mora, P., Garrigues, P., Budzinski, H., Raoux, C., and Narbonne, J. F. 2001. Cytochrome P450 dependent activities in mussel and fish from coastal marine environment: Field studies on the French coast of the Mediterranean Sea. Polycyclic Aromatic Compounds, 18: 307-324.

Myers, M.S., Johnson, L.L., Collier, T.K. 2003. Establishing the causal relationship between polycyclic aromatic hydrocarbon (PAH) exposure and hepatic neoplasms and neoplasia-related liver lesions in English sole (*Pleuronectes vetulus*). Hum Ecol Risk Assess, 9: 67- 94.

Payne, J.F. 1976. Field evaluation of benzopyrene hydroxylase induction as a monitor for marine petroleum pollution. Science, 191: 945-946.

Peters, L.D., Nasci, C., and Livingstone, D.R. 1998. Variation in levels of cytochrome P4501A, 2B, 2E, 3A and 4A- immunopositive proteins in digestive gland of indigenous and transplanted mussel *Mytilus galloprovincialis* in Venice Lagoon, Italy. Marine Environmental Research, 46: 295-299.

Ruus, A., Hylland, K., Green, W.N. 2003. Joint Assessment and Monitoring Programme (JAMP). Biological Effects Methods, Norwegian Monitoring 1997-2001. Norwegian State Pollution Monitoring Programme Report no. 869/03. Ta-no. 1948/2003.

Ruus, A., Sandvik, M., Ugland, K.I., and Skaare, J.U. 2002. Factors influencing activities of biotransformation enzymes, concentrations and compositional patterns of organochlorine contaminants in members of a marine food web. Aquatic Toxicology, 61: 73-87.

Shaw, J.P., Large, A.T., Chipman, J.K., Livingstone, D.R., and Peters, L.D. 2000. Seasonal variation in mussel *Mytilus edulis* digestive gland cytochrome P4501A-and 2E-immunoidentified protein levels and DNA strand breaks (Comet assay). Marine Environmental Research, 50: 405-409.

Sleiderink, H., Beyer, J., Everaarts, J.M., Boon, J.P. 1995. Influence of temperature on cytochrome P450 1A in dab (*Limanda limanda*) from the southern North Sea: Results from field surveys and a laboratory study. Mar. Environ. Res., 39: 67-71.

Sole, M., Peters, L.D., Magnusson, K., Sjolin, A., Granmo, A., and Livingstone, D.R. 1998. Responses of the cytochrome P450-dependent monooxygenase and other protective enzyme systems in digestive gland of transplanted common mussel (*Mytilus edulis L.*) to organic contaminants in the Skagerrak and Kattegat (North Sea). Biomarkers, 3: 49-62.

Stagg, R., and McIntosh, A. 1998. Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity. ICES Techniques in Marine Environmental Science, 23. 16 pp.

Whyte, J.J., Jung, R.E., Schmitt, C.J., and Tillitt, D.E. 2000. Ethoxyresorufin-O-deethylase (EROD) activity in fish as a biomarker of chemical exposure. Critical Reviews in Toxicology, 30: 347-570.

Williams, R.T. 1974. Inter-species variations in the metabolism of xenobiotics. Biochem. Soc. T., 2: 359-377.

Williams, T.G., Lockhart, W.L., Metner, D.A., and Harbicht, S. 1997. Baseline studies in the Slave River, NWT, 1990-1994.3. MFO enzyme activity in fish. Science of the Total Environment, 197: 87-109.

Table 1. EROD Assessment criteria in fish target species used in biomonitoring programmes around European waters. EROD BRs established are restricted to the sampling conditions and the size 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 comes available.

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

Table 2. Description of data used in setting background and elevated response ranges

EROD Assessment *Criteria* *† sub-fraction S9* * microsomes sub-fraction	**Sampling season**	**Bottom water temperature range**	**Size length**	**Sex**	**EROD BR activity** Median (pmol/min/mg prot)	**Upper limit of EROD BR activity** P90 (pmol/min/mg prot)	**N**
		ºC	cm				
Dab (*Limanda Limanda*)	August-November	[10-18]	12-25	Females and/or males	< 30†	< 152†	1034
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	<3.71†	<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 3. Dose-response, background response and sensitivity in experimental studies with gadoid fish

species	substance(s)	lowest-highest concs	exposure time	baseline/control (level/activity)	induction (fold)	reference
Polar cod *Boreogadus saida* juvenile	Crude oil (Oseberg C)	~200 mg/kg (i.p. inj.)	10 and 21 d post inj.	~30 pmol/min/mg	~8 and ~2.5 (245 and 80 pmol/min/mg)	(George *et al*. 1995)
Polar cod *Boreogadus saida* male	Crude oil (Oseberg C)	~200 mg/kg (oral)	21 d post exposure	28 pmol/min/mg ± 6 (n=12)	~5 (132 ± 14 pmol/min/mg)	(George *et al*. 1995)
Polar cod *Boreogadus saida* female	Crude oil (Oseberg C)	~200 mg/kg (oral)	21 d post exposure	8 pmol/min/mg ± 2 (n=14)	~5 (42 ± 6 pmol/min/mg)	(George *et al*. 1995)
Polar cod *Boreogadus saida* juvenile	β-naphthoflavone	50 mg/kg (i.p. inj.)	21 d post inj.	~30 pmol/min/mg	~12.5 (380 pmol/min/mg)	(George *et al*. 1995)
Cod *Gadus morhua* juvenile	2,3,7,8-TCDD	0.008 mg/kg 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/mg	~4 and ~3 (230 and 277 pmol/min/mg)	(Hektoen *et al*. 1994)
Cod, *Gadus morhua* juvenile	PCB-105	10 mg/kg 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/mg	1.5 and 1.2	(Bernhoft *et al*. 1994)
Cod, *Gadus morhua* juvenile	β-naphthoflavone	100 mg/kg (i.p. inj. at d 0 and d 4)	measure at d 7	84 pmol/min/mg ± 8 (n=5)	~13 (1074 ± 340 pmol/min/mg)	(Goksoyr *et al*. 1987)
Cod, *Gadus morhua*	β-naphthoflavone	100 mg/kg (2 i.p. inj.)	measure 3-4 d after last injection	40 pmol/min/mg	~72 (2870 pmol/min/mg)	(Goksoyr *et al*. 1991)
Cod, *Gadus morhua* juvenile	Crude oil (North Sea)	0.06 – 1 ppm	30 days	~2 pmol/min/mg	~ 2 - 5.5 (~ 4 – 11 pmol/min/mg)	(Aas *et al*. 2000)

Table 4. Dose-response, background response and sensitivity in field studies with gadoid fish

Species	**Substance(s)**	**Lowest-highest concs**	**Exposure time**	**Baseline/control (level/activity)**	**Induction (fold)**	**Reference**
Rockling *(Ciliata mustella)*	Crude oil (Gullfaks; M.V. Braer spill, Shetland)	85000 tons spill 129 ± 38 ng/g dry wt. of PAHs (selected 2- and 3-ring) detected in muscle.	3 months after spill	~160 pmol/min/mg ±50	~9 (1480 pmol/min/mg)	(George *et al*. 1995)
Roundnose grenadier (*Coryphaenoides rupestris)*	*i.e.* PAHs and PCBs			260 ± 20 (male) ~170 (female) pmol/min/mg	~2 (530 ± 70 (male) and ~350 (female) pmol/min/mg)	(Lindesjoo *et al*. 1996)
Hake (*Urophycis spp).*	Pollution (PAH) from oil platforms (Gulf of Mexico) <100m from platforms			10.9 ± 6.4 and 11.7 ± 10.5 pmol/min/mg (>3000 m from platforms)	<1 (10.6 ± 3.8 and 10.5 ± 7.1 pmol/min/mg)	(McDonald *et al*. 1996)

Chapter 4: DNA adducts

Background

In the chemical carcinogenesis model the initiating step is the covalent modification of DNA by a carcinogen. The measurement of covalent structures formed between environmental carcinogens and DNA, termed DNA adducts, can be utilised 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:

a) 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.

b) DNA adduct levels integrate multiple toxicokinetic factors such as uptake, metabolisms, detoxification, excretion and DNA repair in target tissues.

c) DNA adducts are relatively persistent once formed (may last several months) and 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.

d) 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). For example, liver and skin neoplasia in brown bullheads (*Ictaluvus nebulosus*) from the Black River, Ohio (USA) have been shown to be strongly correlated with PAH sediment contamination. 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 adduct induced by environmental genotoxins (for a review see Beach and Gupta, 1992; Phillips, 1997; Phillips, 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 assays sensitivity, coupled with the assays ability to detect a wide range of carcinogens (e.g. PAHs), has led

to its wide spread 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.,* 2004b; 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).

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 their 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; Lyons *et al.,* 1999; Lyons *et al.,* 2000; Ericson *et al.,* 2002; Aas *et al.,* 2003; Akcha *et al.,* 2004; Lyons *et al.,* 2004a,b 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 are 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). While 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.,* 1995).

Species selection and target tissue

The majority of hydrophobic genotoxins, such as PAHs, released into the marine environment quickly adhere or 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-postblabelling assay is that it is not species-specific and therefore can be utilised on any organism deemed fit for 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 the levels of DNA adducts were due to 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 infers a chemical aetiology for many of the commonly observed hepatic lesions seen in wild fish collected from contaminated areas. Laboratory data supporting this association stems from biochemical and molecular studies which have revealed the liver to be the major site for contaminant detoxification pathways (e.g. cytochrome P-450-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-postabelling assay is that it is not tissue specific and 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 glad (Ericson *et al.,* 1999; French *et al.,* 1996; Lyons *et al.,* 1997; Harvey and Parry, 1998).

Methodology and technical considerations

32P-postlabelling

In the ^{32}P-postlabeling method, DNA isolated from tissue is first hydrolysed enzymatically to 3'-monophosphates. The proportion of adducts in the enzyme hydrolysate are enriched by selective removal of unmodified nucleotides by enzymatic methods (Reddy and Randerath, 1986) or by extracting the adducts into n-butanol 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 1 adduct in 10^9–10^{10} bases (Reichert *et al.,* 1999). Following the adduct enrichment step, the 3'-monophosphates are radio-labelled at the 5'-hydroxyl using ^{32}P-ATP and T4-polynucleotide kinase to form 3', (^{32}P)5'-bisphosphates. Separation of the ^{32}P-labeled 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).

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 days. 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 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 organisation 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, 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 they should be regularly checked for radioactivity by passing them under a radiation monitor. Gloves should immediately be changed and discarded if found to be 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, the workers are to check themselves and their equipment with the radiation monitor. If any radioactivity is detected then they are to wash themselves and/or the equipment until free of radioactivity.

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 boxes. 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.

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 1994–1997 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).

Assessment criteria

It is recognised that setting 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 a biomarker 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 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 1B), which is a composite of multiple overlapping PAH-DNA adducts.

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

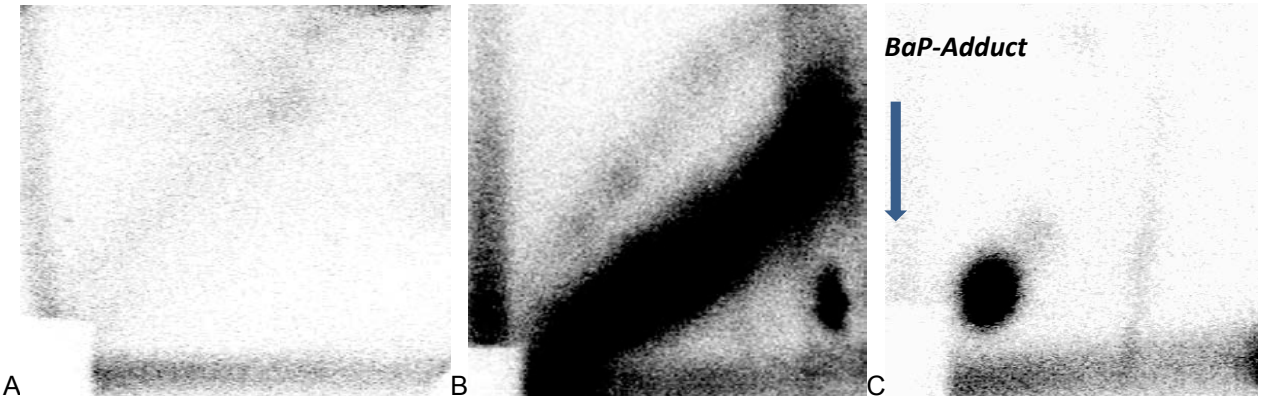

Figure 1 Representative hepatic DNA adducts profiles produced following ^{32}P-postlabelling. (A) DNA adduct profile obtained from a site with a low level of PAH contamination. A faint DRZ is visible, indicating a low level of DNA adducts representative of a clean reference location. (B) DNA adduct profile 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 BaP labelled DNA (115 nucleotides per 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).

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

The determined 90 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; 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). This data has 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 North East Atlantic relevant species herring and halibut exposed to oil (Ingvarsdottir *et al.,* in prep.).

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

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

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 (ERL) value is defined as the lower tenth percentile of the effect. Data were available from Norway (IRIS and NIVA) for other species (IRIS database; BioSea project – Total E&P Norge & Eni Norge); data were reported as nmol adducts/mol DNA. The UK expressed results as adducted nucleotides per 108 normal nucleotides, which was converted to nmol adducts/mol DNA by dividing by 10.

The derived values for dab and flounder were ER-L 1.0 (background), and for Atlantic cod it was 1.6 (background) and for haddock (Barents Sea) it was 3.0 (subtracting a species-specific spot). Threshold value assigned for significant effects in Atlantic cod was 6 (see p.13 above for method of estimation). This value is also indicative for flatfish (to be verified).

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 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)

Concluding remarks

- *DNA adducts as biomarkers of genotoxic exposure*. DNA adducts provide a measure of biologically active contaminant 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 32P, which is an energetic beta emitter. This requires specialist laboratories 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-postblabelling 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-postlabeling method. However, inter laboratory 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, Atlantic cod. In addition, background criteria have been set for haddock and long rough dab. 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 becomes available through national monitoring programmes and through the activities of ICES WGBEC.

References

Aas, E., Liewenborg, B., Grøsvik, B.E., Campus, L., Jonsson, G., Børseth, J.F., Balk, L. 2003. DNA adduct levels in fish from pristine areas are not detectable or low when analysed using the nuclease P1 version of the ^{32}P-postlabelling technique. Biomarkers, 8 (6), 445–460.

Akcha, F., Leday, G., Pfohl-Leszkowicz, A. 2004. Measurement of DNA adducts and strand breaks in dab (*Limanda limanda*) collected in the field: effects of biotic (age, sex) and abiotic (sampling site and period) factors on the extent of DNA damage. Mutation Research 552(1–2):197–207.

Anderson, J.W., Dixit, D.B., Ward, G.S. and Foster, R.S. 1977. Effects of petroleum hydrocarbons on the rate of heart beat and hatching success of estuarine fish embryos. In Vernberg FJ, Calabrese A, Thurnberg FP, Vernberg WB, eds. *Physiological responses of marine biota to pollutant*. Acad. Press, New York, USA, pp 241–258.

Balk, L., Liewenborg, B., Larsen, B.K., Aas, E., Hylland, K., Sanni, S. 2006. Large hydrophobic adducts and strand breaks analysed in hepatic DNA from Atlantic cod (*Gadus morhua*) caged at the Statfjord oilfield. In Biological effects of contaminants in pelagic ecosystems. Editors Hylland K, Lang T, Thain J, Vethaak AD, Wosniok W. Brussels: Society of Environmental Toxicology and Chemistry (SETAC), 277–295.

Bauman, P.C. 1998. Epizootics of cancer in fish associated with genotoxins in sediment and water. Mutation Research 411, 227–233.

Beach, A.C., Gupta, P.C. 1992. Human biomonitoring and the ^{32}P-postlabelling assay. Carcinogenesis 13, 1053–1074.

Castegnaro, M. Bresil, H., Manin, J.P. 1993. Some safety procedures for handling ^{32}P during postlabelling assays, In Postlabelling Methods for Detection of DNA Adducts. Ed. D.H. Phillips, M. Castegnaro & H. Bartsch. Lyon, International Agency for Research on Cancer. IARC Scientific Publications 124, 87–92.

Ericson, G., Lindesjoo, E., Balk, L. 1998. DNA adducts and histopathological lesions in perch (*Perca fluviatilis*) and northern pike (*Esox lucius*) along a polyclic aromatic hydrocarbon gradient on the Swedish coastline of the Baltic Sea. Canadian Journal of Fisheries and Aquatic Sciences, 55, 815–824.

Ericson, G., Noaksson, E., Balk, L. 1999. DNA adduct formation and persistence in liver and extrahepatic tissues of northern pike (*Esox lucius*) following oral exposure to benzo[a]pyrene, benzo[k]fluranthene and 7H-dibenzo[c,g]carbazole. Mutation Research, 427, 136–145.

Ericson, G., Skarphedinsdottir, H., Zuanna, L.D., Svavarsson, J. 2002. DNA adducts as indicators of genotoxic exposure in indigenous and transplanted mussels, *Mytilus edulis* L. from Icelandic coastal sites. Mutation Research 516(1–2):91–9.

French, B.L., Reichart, W.L., Hom, T., Nishimoto, M., Sanborn, H.R., Stein, J.E. 1996. Accumulation and dose-response of hepatic DNA adducts in English sole (*Pleuronectes vetulus*) exposed to a gradient of contaminated sediments. Aquatic Toxicology, 36 (1–2), 1–16.

Gupta, R.C., Reddy, M.C., Randerath, K. 1982. ^{32}P-postlabelling analysis of non-radioactive aromatic carcinogen DNA adducts. Carcinogenesis, 3, 1081–1092.

Harvey, J.S., Parry, J.M. 1998. The analysis of DNA adduct formation, removal and persistence in the common mussel *Mytilus edulis* exposed to 4-nitroquinoline 1-oxide. Mutation Research, 399, 31–42.

IARC. 1993. Postlabelling methods for Detection of DNA adducts. Edited by D,H. Phillips, M. Castegnaro, H. Barysch. IARC Scientific Publications, Lyon (1993) No. 124. 392 pages.

Lyons, B.P., Harvey, J.S., Parry, J.M. 1997. An initial assessment of the genotoxic impact of the Sea Empress oil spill by the measurement of DNA adduct levels in the intertidal teleost *Lipophrys pholis*. Mutation Research, 390, 263–268.

Lyons, B.P., Stewart, C., Kirby, M.K. 1999. The detection of biomarkers of genotoxin exposure in the European flounder (*Platichthys flesus*) collected from the River Tyne Estuary. Mutation Research, 446, 111–119, p116.

Lyons, B.P., Stewart, C., Kirby, M.K. 2000. ^{32}P-postlabelling analysis of DNA adducts and EROD induction as biomarkers of genotoxin exposure in dab (*Limanda limanda*) from British coastal waters. Marine Environmental Research 50 (2000) 575–579.

Lyons, B.P., Bignell, J., Stentiford, G.D., Feist, S.W. 2004a. The viviparous blenny (*Zoarces viviparus*) as a bioindicator of contaminant exposure: application of biomarkers of apoptosis and DNA damage. Marine Environmental Research, 58, 757–762.

Lyons B.P., Stentiford, G.D., Green, M., Bignell, J., Bateman, K., Feist, S.W., Goodsir, F. Reynolds, W.J. Thain, J.E. 2004b. DNA adduct analysis and histopathological biomarkers in European flounder (*Platichthys flesus*) sampled from UK estuaries. Mutation Research, 552, 177–186.

Malins, D.C., Krahn, M.N., Myers, M.S., Rhodes, L.D., Brown, D.W., Krone, C.A., McCain, B.B., Cham, S. 1985. Toxic chemicals in sediments and biota from a creosote-polluted harbour. Relationships with hepatic neoplasms and other hepatic lesions in English sole (*Parophyrs vetulus*). Carcinogenesis, 6, 1463–1469.

Myers, M.S., Landahl, J.T., Krahn, M.M., McCain, B.B. 1991. Relationships between hepatic neoplasms and related lesions and exposure to toxic chemicals in marine fish from the U.S. West Coast. Environmental Health Perspectives, 90, 17–26.

Phillips, D.H., Castegnaro, M. 1993. Results of an interlaboratory trail of ^{32}P-postlabelling. In Postlabelling Methods for Detection of DNA Adducts. Ed. D.H. Phillips, M. Castegnaro & H. Bartsch. Lyon, International Agency for Research on Cancer. IRAC Scientific Publications 124, 35–50.

Phillips, D.H., Castegnaro, M. 1999. Standardization and validation of DNA adduct postlabelling methods: report of interlaboratory trails and production of recommended protocols. Mutagenesis 14 (3), 301–315.

Phillips, D.H. 2005. DNA adducts as markers of exposure and risk. Mutation Research 577, 284–292.

Phillips, D.H. 1997. Detection of DNA modifications by the ^{32}P-postlabelling assay. Mutation Research, 378, 1–12.

Reddy, M.V., Randerath, K. 1986. Nuclease-P1 mediated enhancement of sensitivity of the ^{32}P-postlabelling test for structurally diverse DNA adducts. Carcinogenesis 9, 1543–1551.

Reichert, W.L., Myers, M.S., Peck-Miller, K., French, B., Anulacion, B.F., Collier, T.K., Stein, J.E., Varanasi, U. 1998. Molecular epizootiology of genotoxic events in marine fish: Linking contaminant exposure, DNA damage, and tissue-level alterations. Mutation Research, 411, 215–255.

Reichert, W.L., French, B.L,. Stein, J.E. 1999. Biological effects of contaminants: Measurements of DNA adducts in fish by ^{32}P-postlabelling. ICES Techniques in Marine Environmental Sciences. No. 25.

Richard J. Albertini, R.J., Diana Anderson, D Dougla, D.R., Hagmar, L., Hemminki, K, Merlo, F., Natarajan, A.T., Norppa, H., Shuker, D.E.G., Tice, R., Waters, M.D., Aitio, A. 2000. IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. Mutation Research-Reviews in Mutation Research. 463 (2), 111–172.

Sikka, H.C., Steward, A.R., Kandaswami, C., Rutkowski, J.P., Zaleski, J., Earley, K., Gupta, R.C. 1991. Metabolism of benzo[a]pyrene and persistence of DNA adducts in the brown bullhead (*Ictalurus nebulosus*). Comparative Biochemistry and Physiology, 100c, 25–28.

Scholten, M.C.Th, Schobben, H.P.M, Karman, C.C., Jak, R.G. and Van het Groenewoud, H. 1993. De berekening van het maximal toetlaatbaar risico niveau van olie en oliecomonenten in water en sediment. Technical report R93/187, Dutch Organisation for Applied Scientific Research (TNO), Den Helder, The Netherlands.

Skadsheim, A. 2004. Cod stocks exposed to crude oils: uptake, metabolism and biomarker effects. Technical report AM-2004/005, Akvamiljo, Randaberg, Norway.

Skadsheim, A., Sanni, S., Pinturier, L., Moltu, U.-E., Buffagni, M. and Bracco, L. 2009. Assessing and monitoring long-range transported hydrocarbons as potential stressors to fish stocks. Deep Sea Research Part II. Vol. 56, Issues 21–22, pp. 2037–2043.

Sleiderink, H.M., Beyer, J., Scholtens, E., Goksyr, A., Nieuwenhuize, J., Van Liere, J.M., Everaarts, J.M., Boon, J.P. 1995. Influence of temperature and polyaromatic contaminants on CYP1A levels in North Sea dab (*Limanda limanda*). Aquatic Toxicology, 32: 189–209.

Smit, M.G.D, Bechmann, R.K., Hendriks, A.J., Skadsheim, A., Larsen, B.K., Baussant, T., Bamber, S. and Sanni, S. 2009. Relating biomarkers to whole-organism effects using species sensitivity distributions: A pilot study for marine species exposed to oil. Environmental Toxicology and Chemistry, Vol. 28, No. 5, pp. 1104–1109.

Stein, J.E., Reichert, W.L., Nishimoto, M., Varanasi, U. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology II: Biochemical studies. Science of the Total Environment, 94: 51–69.

Van der Oost, R., Van Schooten, F.J., Ariese, F., Heida, H. 1994. Bioaccumulation, biotransformation and DNA binding of PAHs in feral eel (*Anguilla anguilla*) exposed to polluted sediments. Environmental Toxicology and Chemistry, 13: 859–870.

Chapter 5: Externally visible fish diseases, macroscopic liver neoplasms and liver histopathology)

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 BEQUALM (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 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 organisation. Certain types of non-neoplastic and neoplastic liver lesions (as specified in the guidelines for the JAMP/CEMP) are known to be associated with prior exposure to carcinogenic contaminants such as PAHs.

Assessment of thresholds

For externally visible diseases Background Assessment Criteria (BAC) and Environmental Assessment Criteria (EAC) have been defined. For non-specific liver histopathology, BACs have been defined (*EAC awaiting actual ICES data*). Additionally, significant changes in disease prevalence levels and trends serve as a basis for threshold assessments. For macroscopic liver neoplasms and contaminant-specific liver histopathology, assessment criteria have been proposed by the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC) (*modification possible, depends on actual ICES data*) (ICES 2009b).

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 meeting. At the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC), assessment criteria for macroscopic liver neoplasms and for contaminant-specific 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.

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, in particular 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 Commission, 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 2 and Annex 1).

At present, annual or biannual fish disease surveys in the North Sea are carried out by Germany (vTI, Inst. of Fishery Ecology, Cuxhaven), The Netherlands (RIKZ) and the UK (Cefas, Weymouth; Marine Scotland, Aberdeen). However, more data is 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 south-western Baltic Sea. The major target fish species in the North Sea is dab (*Limanda limanda*), in the Baltic Sea flounder (*Platichthys flesus*) and cod (*Gadus morhua*). 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 UK National Marine Monitoring Programme (NMMP) 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. 10–15 offshore areas are 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.

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 (e.g. 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 Chapter 'Proposals for assessment tools').

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 standardised 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 practical ICES sea-going workshops on board research vessels were organised 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 standardise methodologies for fish disease surveys (Dethlefsen *et al*., 1986; ICES, 1989, 2006a; Lang and Mellergaard, 1999) and to prepare guidelines. Whilst 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 standardisation 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 1).

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 Marine Data Centre has been formalised by the introduction of the ICES Environmental Reporting Format designed specifically for the 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 comprise mainly 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).

Table 1. 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
	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

Current ICES WGPDMO activities have focussed 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 analyses 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 are 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.

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 can 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 organisation (molecule, sub-cellular 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 the temporal changes in disease prevalence in the North Sea. While data from the 1990s revealed stable or decreasing disease prevalences 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 of 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 to 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, providing strong supporting evidence that wild fish exhibiting these lesions could have been exposed to such environmental contaminants.

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 minimise 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 (see below) provides clear criteria to discriminate between the lesion types, thus minimising the possibility of mis-diagnosis. Ring tests and other intercalibration exercises are regularly undertaken in order to minimise inter-observer 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 (see below) 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 Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC) addressed the question of establishing background/reference levels of disease and criteria for their assessment (see Chapter ‘Proposals for assessment tools’).

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 Working Group on Pathology and Diseases of Marine Organisms (WGPDMO) (ICES, 2006b, 2007, 2008, 2009a, 2011), Further additions were proposed at the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (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 summarise 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, categorised as externally visible diseases (EVD: nine key diseases, incl. three parasites), macroscopic liver neoplasms (MLN: two key diseases) and liver histopathology (LH: five key diseases) (see Table 2);
- For most diseases, data on three severity grades (reflecting a light, medium or severe disease status) are included;
- 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.

Table 2 Disease categories and key diseases to be used for calculating the Fish Disease Index for dab (*Limanda limanda*) (ICES 2009a)

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	Benign neoplasms
Epidermal hyperplasia/papilloma		toxicopathic lesions	Malignant neoplasms
Acute/healing skin ulceration		Pre-neoplastic lesions	
X-cell gill disease		(FCA)	
Hyperpigmentation		Benign neoplasms	
Acute/healing fin rot/erosion		Malignant neoplasms	
Stephanostomum baccatum			
Acanthochondria cornuta			
Lepeophtheirus pectoralis			

The result of the calculation is a FDI value for individual fish which is scaled in 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. All assessment is 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 years' 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-wide basis, global BAC and EAC are used by 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 implausible 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, 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.

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; and
- 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 5-years 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);
- maps in which the geographical units assessed are marked with green, yellow or red smiley faces, indicating the level of the FDI for external diseases observed at a defined point in time (green: below the BAC; yellow: between BAC and EAC; red: above the EAC, reason for concern and motivation for further research on causes);
- maps in which the geographical units assessed are marked with green or red smiley faces, indicating the level of the FDI for macroscopic neoplasms observed at a defined point in time (green: below the BAC; red: above the EAC, reason for concern and motivation for further research on causes).

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, 2009b). The results will be included in the OSPAR QSR 2010 as a case study.

At the 2009 ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC), additional assessment criteria for macroscopic liver neoplasms and for the contaminant-specific components of liver histopathology were proposed. These are provided in Table 3, which also contains the BAC and EAC for the FDI-EVD, which had been agreed upon at the 2011 meeting of the WGPDMO.

Table 3. Assessment criteria proposed for the assessment of contaminant-specific effects on fish health (Note: the colour 'red' should be used for graphical representations of the categories 'elevated response/above background' as well as for 'significant response/unacceptable effects' in maps or similar illustrations).

Disease category	Background	Elevated response/ above background	Significant response/unacceptable effects
Externally visible diseases (to be used as additional information for the assessment)	see Table 4	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period or Statistically significant upward trend in mean FDI level in the assessment period or BAC ≤ FDI level < EAC	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period or Statistically significant upward trend in mean FDI level in the assessment period or EAC ≤ FDI level
Liver histopathology: non-specific (to be used as additional information for the assessment)	Not applicable	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period or Statistically significant upward trend in mean FDI level in the assessment period	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period or Statistically significant upward trend in mean FDI level in the assessment period
Liver histopathology: contaminant-specific	Mean FDI < 2	Mean FDI ≥ 2 A value of 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.	Mean FDI ≥ 2 A value of 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	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.	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.

Table 4. Assessment criteria for the assessment of the FDI for externally visible diseases in common dab (*Limanda limanda*). Abbreviations used: 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.

Sex	**Diseases/ parasites involved in FDI (see legend for abbreviations)**	**Background Assessment Criteria**		**Environmental Assessment Criteria**	
		ungraded diseases	graded diseases	ungraded diseases	graded diseases
F	Ep, Ly, Ul	1.32	0.216	-	54.0
M	Ep, Ly, Ul	0.96	0.232	-	47.7
F	Ac, Ep, Fi, Hp, Le, Ly, St, Ul, Xc	1.03	0.349	50.6	19.2
M	Ac, Ep, Fi, Hp, Le, Ly, St, Ul, Xc	1.17	0.342	38.8	16.1
F	Ac, Ep, Hp, Le, Ly, St, Ul, Xc	1.09	0.414	48.3	21.9
M	Ac, Ep, Hp, Le, Ly, St, Ul, Xc	1.18	0.398	35.2	16.5

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.

Reference

Bucke, D., Vethaak, A. 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, 19. 27 pp.

Dethlefsen, V., Egidius, E., and McVicar, A. H. 1986. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV ‘Anton Dohrn’ 3-12 January 1984. ICES Cooperative Research Report, 140. 33 pp.

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 Science, 28: 47pp.

HELCOM 1996. Third Periodic Assessment of the State of the Marine Environment of the Baltic Sea, 1989-1993; Background Document. Baltic Sea Environment Proceedings N0 64 B. 252 pp.

HELCOM 2002. Environment of the Baltic Sea area 1994-1998. Baltic Sea Environment Proceedings N0 82 B. 215 pp.

ICES 1989. Methodology of fish disease surveys. Report of an ICES Sea-going Workshop held on RV U/F 'Argos' 16–23 April 1988. ICES Cooperative Research Report, 166. 33 pp.

ICES 1997. Report of the Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants. ICES CM 1997/F:2. 75 pp.

ICES 2005. ICES Advice http://www.ices.dk/committe/acfm/comwork/report/2005/may/Technical%20Annex%20to%20the%20review%20of%20WKIMON.pdf.

ICES 2006a. Report of the ICES/BSRP Sea-going Workshop on Fish Disease Monitoring in the Baltic Sea (WKFDM). ICES CM 2006, BCC:02, 85 pp.

ICES 2006b. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2006/MCC:01, 104 pp.

ICES 2007. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2007/MCC:04, 92 pp.

ICES 2008. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2008/MCC:01, 128 pp.

ICES 2009a. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2009/MCC:01, 111 pp.

ICES 2009b. Report of the ICES/OSPAR Workshop on Assessment Criteria for Biological Effects Measurements (WKIMC), ICES CM 2009/ACOM:50, 133 pp.

ICES 2011. Report of the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO), ICES CM 2011/SSGHIE:04.

Lang, T. 2002. Fish disease surveys in environmental monitoring: the role of ICES. ICES Marine Science Symposia, 215: 202–212.

Lang, T., and Mellergaard, S. 1999. The BMB/ICES Sea-Going Workshop 'Fish Diseases and Parasites in the Baltic Sea' – introduction and conclusions. ICES Journal of Marine Science, 56: 129–133.

Lang, T., and Wosniok, W. 2000. ICES data for a holistic analysis of fish disease prevalence. In ICES 2000, Report of the ICES Advisory Committee on the Marine Environment, 1999, pp. 193–209. ICES Cooperative Research Report, 239. 277 pp.

Lang, T. and Wosniok, W. 2008. The Fish Disease Index: a method to assess wild fish disease data in the context of marine environmental monitoring. ICES CM 2008/D:01, 13 pp.

Myers, M.S., Rhodes, L.D. and McCain, B.B. 1987. Pathologic anatomy and patterns of occurrence of hepatic neoplasms, putative preneoplastic lesions, and other idiopathic conditions in English sole (*Parophyrs vetulus*) from Puget Sound, Washington. Journal of the National Cancer Institute 78, 333–363.

Myers, M.S., Olson, O.P., Johnson, L.L., Stehr, C.M., Hom, T., and Varanasi, U. 1992. Hepatic lesions other than neoplasms in sub-adult flatfish from Puget Sound, WA: Relationships with indices of contaminant exposure. Mar. Environ. Res. 34, 45–51.

Myers, M.S., Johnson, L.L., Hom, T., Collier, T.K., Stein, J.E., and Varanasi, U. 1998. Toxicopathic lesions in sub-adult English sole (*Pleuronectes vetulus*) from Puget Sound, Washington, USA: Relationships with other biomarkers of contaminant exposure. Marine Environmental Perspectives, 45, 47–67.

North Sea Task Force 1993. North Sea Quality Status Report 1993. Oslo and Paris Commissions, London. Olsen and Olsen, Fredensborg, Denmark. 132 pp.

OSPAR Commission 2000. Quality Status Report 2000, Region II-Greater North Sea. OSPAR Commission, London. 136+xiii pp.

Stein, J.E., Reichert, W.L., Nishimito, M., and Varanasi, U. 1990. Overview of studies on liver carcinogenesis in English sole from Puget Sound; evidence for a xenobiotic chemical etiology, 2, Biochemical studies. Sci. Total Environ. 94, 51–69.

Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M. and Feist, S.W. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Marine Environmental Research 55, 137–159.

Vethaak, A.D. and Wester, P.W. 1996. Diseases of flounder *Platichthys flesus* in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. II. Liver histopathology. Dis. Aquat. Orgs., 26, 81–97.

Wosniok, W., Lang, T., Dethlefsen, V., Feist, S.W., McVicar, A.H., Mellergaard, S., and Vethaak, A.D. 2000. Analysis of ICES long-term data on diseases of North Sea dab (*Limanda limanda*) in relation to contaminants and other environmental factors. ICES CM 2000/S:12. 15 pp.

Wosniok, W., Lang, T., Vethaak, A.D., des Clers, S., and Mellergaard, S. 1999. Statistical analysis of fish disease prevalence data from the ICES Environmental Data Centre. In Report of the ICES Advisory Committee on the Marine Environment, 1998, pp. 297–327. ICES Cooperative Research Report, 233. 375 pp.

Appendix 1: Fish disease monitoring in the OSPAR Coordinated Environmental Monitoring Programme (CEMP) reflecting ICES advice (ICES 2005)

Table 2a. PAH-specific biological effects monitoring.

	Species	Diseases	Numbers	Guidelines
Macroscopic liver neoplasms	Dab (1st 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. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES TIMES No. 19. Relevant in addition: ICES 1989. Methodology of fish disease surveys. ICES Coop. Res. Rep. 166. Feist *et al*., 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. ICES TIMES 38, 42 pp. BEQUALM
	Flounder (Platichthys flesus)		Size group ≥ 30 cm: 50 (if not available in sufficient numbers, include size group 25–29 cm)	
Liver histopathology	Dab (1st 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 Size group 25–29 cm: 50	JAMP Guidelines based on: ICES 1997. Report of the Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of Contaminants. ICES CM 1997/F:2. Relevant in addition: Feist *et al*., 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. ICES TIMES 38, 42 pp. BEQUALM
	Flounder (*Platichthys flesus*)			
	Dragonet (*Callionymus* spp.)		Size group 10–15 cm : 50	No JAMP guidelines so far Relevant: Feist *et al*., 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES TIMES 38, 42 pp.

Table 2b. General biological effects monitoring.

	Species	Diseases	Numbers	Guidelines
Externally visible fish diseases	Dab (1st 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. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES TIMES No. 19.
	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	Relevant in addition: ICES 1989. Methodology of fish disease surveys. ICES Coop. Res. Rep. 166. BEQUALM
	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: 100 Size group 20–29: 100 Size group ≥ 30: 50	No JAMP guidelines so far Relevant: Bucke *et al*., 1996. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES TIMES No. 19.
Macroscopic liver neoplasms	Dab (1st 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. Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES TIMES No. 19. Relevant in addition: ICES 1989. Methodology of fish disease surveys. ICES Coop. Res. Rep. 166. Feist *et al*. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. ICES TIMES 38, 42 pp. BEQUALM
	Flounder (*Platichthys flesus*)		Size group ≥ 30 cm: 50 (if not available in sufficient numbers, include size group 25–29 cm)	
Liver histopathology	Dab (1st priority) (*Limanda limanda*)		Size group 20–24 cm: 50	JAMP Guidelines based on: Bucke *et al*., 1996.

	Species	Diseases	Numbers	Guidelines
	Flounder (*Platichthys flesus*)	Non-specific lesions Early toxicopathic non-neoplastic lesions Foci of cellular alteration Benign neoplasms Malignant neoplasms	Size group 25–29 cm: 50	Common diseases and parasites of fish in the North Atlantic: Training guide for identification. ICES TIMES No. 19. Relevant in addition: ICES 1989. Methodology of fish disease surveys. ICES Coop. Res. Rep.166. Feist *et al*. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. ICES TIMES 38, 42 pp. BEQUALM
	Dragonet (*Callionymus* spp.)		Size group 10–15 cm : 50	No JAMP guidelines so far for Dragonet Relevant: Feist *et al*. 2004. Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda L*.) and flounder (*Platichthys flesus L*.) for monitoring. ICES TIMES 38, 42 pp.

Chapter 6: Histopathology of mussels (*Mytilus spp*.) for health assessment in biological effects monitoring

Background

Mussels have long been used for the measurement of pollutants and the biological effects of contaminants in the aquatic environment (Bayne, 1976; Goldberg, 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 gravely substrates. This makes them well placed as a sentinel species in programmes designed to monitor the marine environment. Over the years numerous studies utilising mussels have demonstrated the impact of anthropogenic inputs into the aquatic environment. Early studies such as the "Mussel Watch" programme (Goldberg, 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 focussed 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 OSPAR Commission (OSPAR) region that incorporate both chemical analysis 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 providing health assessment of individuals and of populations since 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 employed to investigate changes related to PAH, 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 characterisation of toxicopathic pathologies, which are increasingly being used as indicators of environmental stress, in addition to disease.

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 homogenous groups attributes and to control for potential variation amongst individuals). This approach should help 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 ICES TIMES document 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. Whilst the latter may initially seem misplaced in this document describing contaminant induced pathology, it is important to note that disease conditions of pathogen 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.

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

When sampling mussels from 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 into 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 minimised, 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 should be 50 although this may be refined in the final publication of the TIMES document for mussel histopathology (to be published imminently).

The dissection process is an extremely important stage in the histological process and it is crucial that it is conducted in a standardised manner. Standardised 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 as not to cause any damage to any of the tissues. Any damage caused 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 scalpel blade into the mid-ventral 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 as 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 remove 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 previous, 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 whilst 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 standardised 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 due to 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” facilitates this greatly.

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 ten 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 Formalin fixed paraffin embedded (FFPE) histology. Chucks should be transported to the laboratory in dry-ice (if required) and subsequently stored at -80°C.

Histology

Formalin fixed paraffin embedded - 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.

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 3µm to 5 µm and floated onto a pre-heated water bath (35°C–40°C) containing a suitable tissue adhesive (e.g. Sta-On, Surgipath, UK). Alternatively, commercially available slides that have been pre-treated 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 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 (protocol provided in Annex 3) or a suitable alternative. Following staining, the end result should represent Figure X. This approach produces a uniform histological section that (a) incorporates all of the target organs of interest and (b) makes for a more simple microscopic examination due to the standardised 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.

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 organised by Cefas.

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.

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; 2007; Garmendia *et al*., 2011b). 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 (Lowe and Clarke, 1989; Cajaraville *et al*., 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 volume density of basophilic cells (VvBAS) as a measure of digestive cells loss by counting on H/E 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 20x 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:

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 signification 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 $\mu m^3/\mu m^3$

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., through 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. Particularly, 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*., 2011b). 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 (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*., 2011b), 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*., 2011b).

The following table describes 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 about 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 (1996).

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. A total of 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 light microscope using a 20x 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 digitised for data input into 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) are calculated as integrative measures of changes in the alveolar morphology, epithelial thinning included, as follows:

$$MET = 2(Ao-Ai)/(Po+Pi);$$

$$MLR = \sqrt{(Ai/\pi)}; \text{ and}$$

$$MDR = \sqrt{(Ao/\pi)};$$

where Ao is the section area of the whole tubule profile, Po is the perimeter of a circle with area Ao, Ai is the section area of the lumen profile and Pi is the perimeter of the corresponding circle with are Ai. The statistical signification 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 (spring-summer) and 1.2 µm/µm (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 (Marigómez *et al*., 2006).

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 endo-lysosomal 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 and Marigómez 2009a; Izagirre and Marigómez 2009b). 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).

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; Domoutsidou and Dimitriadis, 2001; Garmendia *et al*., 2011a). 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 altogether 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 endo-lysosomal system and is therefore the most used parameter.

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 minutes and then at RT for 5 minutes 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 minutes at 37°C in a shaking water bath. After incubation, slides are rinsed in a 2.5% NaCl solution for 2 minutes 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 minutes in the dark and at RT. Afterwards, the sections are fixed for 10 minutes 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 minutes, 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) 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):

$$VvLYS\ (\mu m^3/\mu m^3) = K \times AA;$$

$$SvLYS\ (\mu m^2/\mu m^3) = (4/t) \times AA;$$

$$S/VLYS\ (\mu m^{-1}) = 4/(t \times K)\ ;\ \text{and}$$

$$NvLYS\ (\mu m^2/\mu m^3) = (4 \times AA \times n) / (t \times \pi \times \Sigma\ Yi2)$$

Being

$$AA = x/m \text{ and } K = (2/(3 \times t))(\Sigma Yi3/\Sigma Yi2);$$

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 (Y1, Y2, … Y90 for n=90).

Lysosomal structural changes test parameters can be tested using analysis of variance. VvLYS and Nv LYS data may need to be logarithmically transformed previous to the statistical analyses since 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 increased 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 Nv LYS, (increased numbers of lysosomes relative to digestive cell cytoplasm) but reductions in Nv LYS 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 e.g. the degree of exposure to pollutants.

Inflammation

Inflammation affects all tissues and organs and is particularly obvious in mussels that have been adversely affected by contaminants (Auffret, 1988; Crouch, 1985). Whilst 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 characterised 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, unrecognised injury or sub-microscopic agents in bivalves. Haemocytic infiltration could be interpreted as a repair process following tissue damage, albeit 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 (BC) 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. Comprised 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 (Marigomez *et al*., 2002). The occurrence of BC aggregates (foci) has been considered an indicator of stress caused by xenobiotics, as well as with age and reproductive stress. BC aggregates are also observed within the gonad follicles following spawning, which is a normal event.

Large foci of inflammation termed granulocytomas (comprised of granulocytes), have previously been seen in mussels of both laboratory and field studies designed to monitor the effects of contaminants. 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).

The following table describes 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	Low numbers of inflammatory foci occupying ≤ 10% of the vesicular connective tissue (approximately 20 small foci) within standardised 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. Increased numbers of Brown cell foci predominately 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. Increased numbers of Brown cell foci predominately within the vesicular connective tissue, stomach and digestive gland epithelium. Increased pigment density.

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 are described in full detail in the ICES TIMES document.

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

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 $\mu m^3/\mu m^3$
	MLR/MET: Digestive tubule epithelial atrophy and thinning (quantitative)	<0.7 µm/µm	1.2–1.6 µm/µm	>1.6 µm/µm
	VVLYS & 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)

References

Auffret, M. 1988. Histopathological changes related to chemical contamination in *Mytilus edulis* from field and experimental conditions. Mar Ecol Prog Ser 46: 101–107.

Bayne, B.L. 1976. Watch on mussels. Mar Pollut Bull 7(12): 217–128.

Bignell, J.P., Dodge, M.J., Feist, S.W., Lyons, B., Martin, P.D., Taylor, N.G.H., Stone, D., Travalent, L., Stentiford, G.D. 2008. Mussel histopathology: effects of season, disease and species. Aquatic Biology 2, 1–15.

Bignell, J.P., Caraville, M., Feist, S.W., Villalba, A., Marigomez, I. (in prep). Histopathology of mussels *Mytilus* sp. for health assessment in biological effects monitoring. ICES Techniques in Marine Environmental Sciences No. XX.

Cajaraville, M.P., Marigómez, J.A., Angulo, E. 1989. A stereological survey of lysosomal structure alterations in Littorina littorea exposed to 1-naphthol. Comp Biochem Physiol 93C: 231–237.

Cajaraville, M.P., Díez, G., Marigómez, I.A., Angulo, E. 1990. Responses of basophilic cells of the digestive gland of mussels to petroleum hydrocarbon exposure. Diseases Aquatic Organisms 9, 221–228.

Cajaraville, M.P., Marigómez, I., Díez, G., Angulo, E. 1992. Comparative effects of the water accommodated fraction of three oils on mussels. 2. Quantitative alterations in the structure of the digestive tubules. Comp. Biochem. Physiol. 102 (C): 113–123.

Cajaraville, M.P., Abascal, I., Etxeberria, M, Marigómez, I. 1995. Lysosomes as cellular markers of environmental pollution: time- and dose-dependent responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Environ Toxicol Water Qual 10:18.

Couch, J. 1984. Atrophy of diverticular epithelium as an indicator of environmental irritants in the oyster *Crassostrea virginica*. Mar. Environ. Res. 14: 525–526.

Couch, J.A. 1985. Prospective study of infectious and non-infectious diseases in oysters and fishes in three Gulf of Mexico estuaries Diseases of Aquatic Organisms 1: 59–82.

Domouhtsidougarmen, G.P., Dimitriadis, V.K. 2001. Lysosomal and lipid alterations in the digestive gland of mussels, *Mytilus galloprovincialis* (L) as biomarkers of environmental stress. Environ Pollut:115:123–137.

Garmendia, L., Izagirre, U., Cajaraville, M.P. and Marigomez, I. 2011a. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and the Bay of Biscay: Lysosomal responses, J. Environ. Monit., DOI: 10.1039/c0em00409j.

Garmendia, L., Soto, M., Vicario, U., Kim, Y., Cajaraville, M.P. and Marigómez, I. 2011b. Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of the Prestige oil spill in Galicia and the Bay of Biscay: Tissue-level biomarkers and histopathology, J. Environ. Monit., DOI: 10.1039/c0em00410c.

Goldberg, E.D., Bowen, V.T., Farrington, J.W., Harvey, G., Martin, J.H., Parker, P.L., Risebrough, R.W., Robertson, W., Schneider, E., Gamble, E. 1978. The Mussel Watch Environ Conserv 5 (2): 101–125.

Granmo, A. 1995. Mussels as a tool in impact assessment. Phuket Mar Biol Cent Spec Publ, 15: 215–220.

Hines, A., Oladiran, G., Bignell, J., Stentiford, G.D., Viant, M.R. 2007. Direct sampling of organisms from the field and knowledge of their phenotype: Key recommendations for environmental metabolomics. Environ Sci Tech 41: 3375–3381.

Izagirre, U., Ramos, R., Marigómez, I. 2008. Natural variability in size and membrane stability of lysosomes in mussel digestive cells: season and tidal zonation. Mar Ecol Prog Ser 372; 105–117.

Izagirre, U., Marigómez, I. Lysosomal enlargement and lysosomal membrane destabilisation in mussel digestive cells measured by an integrative index. 2009a. Environ Pollut; 157: 1544–1553.

Izagirre, U., Angulo, E., Wade, S.C., ap Gwynn, I., Marigómez, I. 2009b. β-Glucuronidase and hexosaminidase are marker enzymes for different compartments of the endo-lysosomal system in mussel digestive cells. Cell and Tissue Research 335: 441–454.

Kim, Y., Ashton-Alcox, A., Powell, E.N. 2006. Histological techniques for marine bivalve molluscs: update NOAA Technical Memorandum NOS NCCOS 27. p. 76.

Kluytmans, J.H., Brands, F., Zandee, D.I. 1988. Interactions of Cadmium with the Reproductive Cycle of *Mytilus edulis* L. Mar Environ Res 24: 189–192.

Langton, R.W. 1975. Synchrony in the digestive diverticula of *Mytilus edulis* L. J. Mar Biol Ass UK 55:221–229.

Lowe, D.M., and Pipe, R.K. 1987. Mortality and quantitative aspects of storage cell utilization in mussels, *Mytilus edulis*, following exposure to diesel oil hydrocarbons. Mar Environ Res 22: 243–251.

Lowe, D.M., Moore, M.N., Clarke, K.R. 1981. Effects of oil on digestive cells in mussels: Quantitative alterations in cellular and lysosomal structure. Aquatic Toxicology 1, Issue 3–4, 213–226.

Lowe, D.M. 1988. Alterations in cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Mar Ecol Prog Ser 46:91–100.

Lowe, D.M., Clarke, K.R. 1989. Contaminant-induced changes in the structure of the digestive epithelium of Mytilus edulis. Aquatic Toxicology 15, Issue 4, 345–358.

Marigómez I, Cajaraville M.P., Angulo, E. 1990. Histopathology of the digestive gland-gonad complex of the marine prosobranch *Littorina littorea* (L) exposed to cadmium. Dis. Aquat. Toxicol. 9: 229–238.

Marigómez I., Soto, M., Etxeberria, M., Angulo, E. 1993. Effects of size, sex, reproduction, and trematode infestation on the quantitative structure of digestive tubules in stressed winkles. Zool. Jb. Anat. 123: 319–336.

Marigómez I., Cajaraville, M.P., Soto, M., Lekube, X. 1998. Cell-type replacement, a successful strategy of molluscs to adapt to chronic exposure to pollutants. Cuad. Invest. Biol. 20: 255–258.

Marigómez I., Soto, M., Cajaraville, M.P., Angulo, E., Giamberini, L. 2002. Cellular and subcellular distribution of metals in molluscs. Micros. Res. Tech. 56: 358–392.

Marigómez, I., Baybay-Villacorta, L. 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquatic Toxicology 64, Issue 3, Pages 235–257.

Marigómez, I., Soto, M., Orbea, A., Cancio, I., Cajaraville, M.P. 2004. Chapter 14: Biomonitoring of environmental pollution in the Basque coast using molecular, cellular and tissue-level biomarkers: an integrative approach. In: Borja, A., Collins, M. (Eds.), Oceanography and marine environment in the Basque Country. Elsevier Oceanography Series N. 70, Elsevier, Amsterdam, pp. 335–364.

Marigómez, I., Izagirre, U., Lekube, .X. 2005. Lysosomal enlargement in digestive cells of mussels exposed to cadmium, benzo[a]pyrene and their combination. Comp Biochem Physiol 2005a; 141:188–193.

Marigómez, I., Soto, M., Cancio, I., Orbea, A., Garmendia, L., and Cajaraville, M.P. 2006. Cell and tissue biomarkers in mussel, and histopathology in hake and anchovy from Bay of Biscay after the Prestige oil spill (Monitoring Campaign). Mar Pollut Bull 53: 287–304.

Moore, M.N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel (*Mytilus edulis*), and changes induced by thermal stress. Cell Tissue Res 175:279–287.

Moore, M.N. 1985. Cellular responses to pollutants. Mar Pollut Bull 16:134–139.

Moore, M.N. 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in mollusc digestive cells to environmental and experimental exposure to xenobiotics. Mar Ecol Prog Ser 46:81–89.

Rasmussen, L.P.D., Hage, E., Karlog, O. 1985. Light and electronmicroscopic studies of the acute and long-term toxic effects of N-nitrosodipropylamine and N-methylnitrosurea on the marine mussel *Mytilus edulis*. Mar Biol 1985; 85: 55–65.

Robinson, W.E. 1983. Assessment of bivalve intracellular digestion based on direct measurement. J. Moll. Stud. 49: 1–8.

Salazar, M.H., Salazar, S.M. 1995. In-situ bioassays using transplanted mussels: I. Estimating chemical exposure and bioeffects with bioaccumulation and growth. ASTM Special Technical Publication, (1218), pp. 216–241.

Soto, M., Zaldibar B., Cancio, I., Taylor M.G., Turner, M., Morgan, J.A., Marigómez, I. 2002. Subcellular distribution of cadmium and its cellular ligands in mussel digestive gland cells as revealed by combined autometallography and X-ray microprobe analysis. Histochem. J. 34: 273–280.

Stentiford, G.D., Viant, M.R., Ward, D.G., Johnson, P.J., Martin, A., Wei, W., Cooper, H.J., Lyons, B.P., Feist, S.W. 2005. Liver tumours in wild flatfish: a histopathological, proteomic, and metabolomic study. Omics: A Journal of Integrative Biology, 9(3): 281–299.

Sunila, I. 1984. Copper- and cadmium- induced histological changes in the mantle of *Mytilus edulis L*. (Bivalvia). Limnologica, 15(2), 523–527.

Vega, M.M., Marigómez I., Angulo E. 1989. Quantitative alterations in the structure of digestive cells of *Littorina littorea* on exposure to cadmium. Mar. Biol. 103: 547–553.

Ward, D.G., Wei W., Cheng Y., Billingham, L.J., Martin, A., Johnson, P.J., Lyons B.P., Feist S.W., Stentiford G.D. 2006. Plasma proteome analysis reveals the geographical origin and liver tumour status of dab (*Limanda limanda*) from UK marine waters. Environ Sci Tech 40: 4031–4036.

Weibel, E.R. 1979. Stereological methods. Vol 1. Acad Press, London, 415 pp.

Widdows, J., and Donkin, P. 1992. Mussels and environmental contaminants: Bioaccumulation and physiological aspects. In: Gosling, The mussel *Mytilus*: Ecology, physiology, genetics and culture. Elsevier, Amsterdam, p 383–464.

Winstead, J.T. 1995. Digestive tubule atrophy in Eastern oysters, *Crassostrea virginica* (Gmelin, 1791), exposed to salinity and starvation stress. J. Shellfish Res. 14: 105–111.

Zaldibar, B., Cancio, I., Marigómez, I. 2007. Reversible alterations in epithelial cell turnover in digestive gland of winkles (*Littorina littorea*) exposed to cadmium and their implications for biomarkers measurements. Aquat. Toxicol. 81: 183–196.

Zorita, I., Ortiz-Zarragoitia, M., Soto, M., Cajaraville, M.P. 2006. Biomarkers in mussels from a copper site gradient (Visnes, Norway): An integrated biochemical, histochemical and histological study. Aquat Toxicol ;78S:S109–S116.

Zorita, I., Apraiz, I., Ortiz-Zarragoitia, M., Orbea, A., Cancio, I., Soto, M., Marigómez, I., Cajaraville, M.P. 2007. Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. Environ Pollut. 148:236–250.

Chapter 7: Intersex (ovotestis) measurement in marine and estuarine fish

Summary

Applicability across OSPAR maritime area

The presence of susceptible host species utilised 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 diseases 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 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 epidemiological relevant numbers of animals is also well characterised 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 impacted 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 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. Since intersex prevalence is likely to be negligible in non-impacted 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 impacted 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 of 4 years old) (Stentiford *et al*., 2010).

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 and De Metrio *et al*., 2003, respectively), and the dab *Limanda limanda* (Scott *et al*., 2007; Stentiford and Feist, 2005, respectively). 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*., 2004), red mullet (Martin-Skilton *et al*., 2006) and the 3-spined stickleback (Gercken and Sordyl, 2002).

Status of quality assurance 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. Due 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: 1. Individual (grading of intersex severity) and 2. Population (intersex prevalence).

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 United Kingdom 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) utilised samples collected from monitoring programs around the United Kingdom 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 to 5 μm, mounted onto glass slides, and stained using haematoxylin and eosin (H and E). Sections were analysed by light microscopy. A total of 56 intersex cases were examined. All gonadal sections were viewed at low magnification using a x10 eyepiece and x10 objective lens, giving a total magnification of x100. 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 2 from Bateman *et al*., 2004 below).

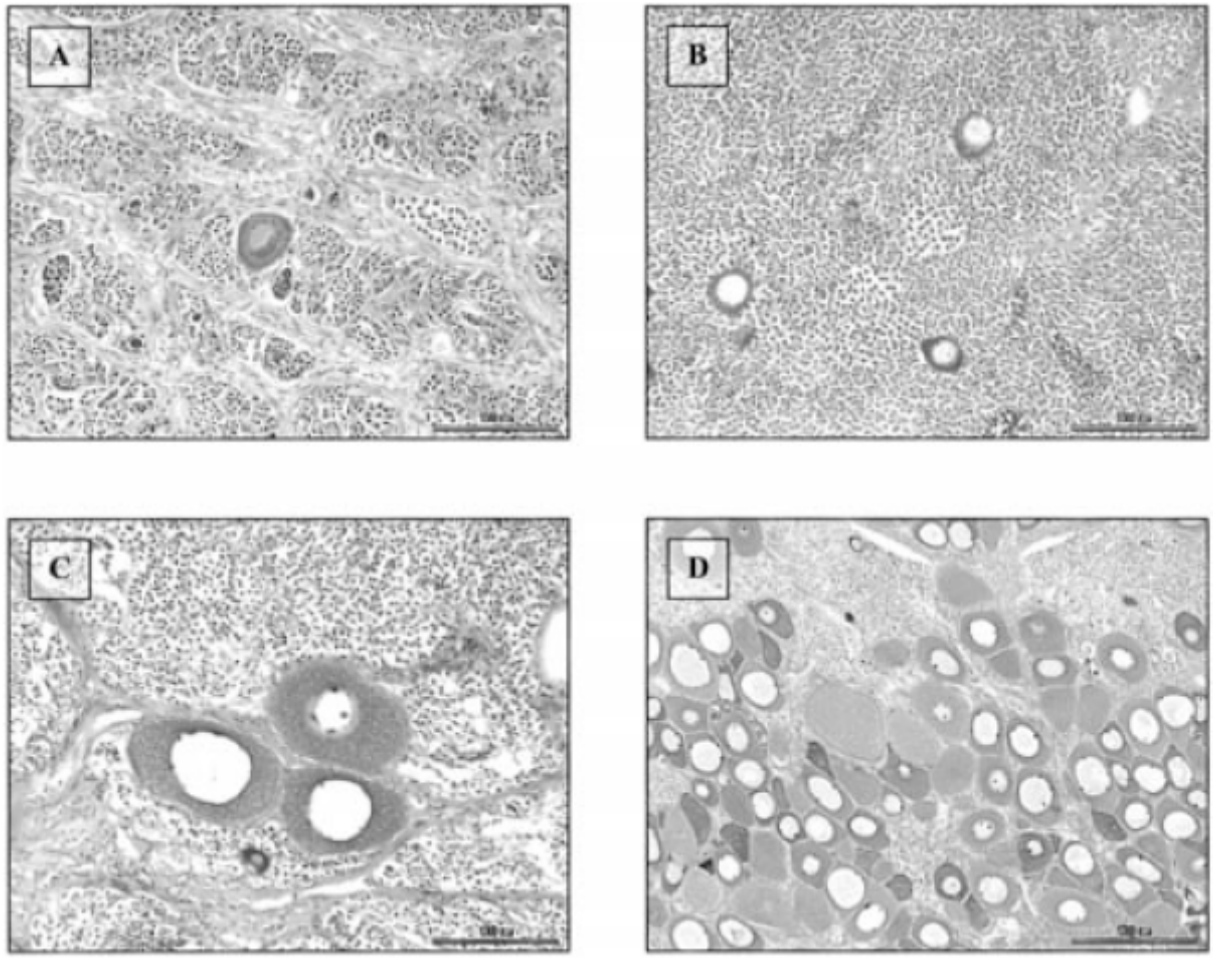

Figure 2. Oocyte distribution patterns in ovotestis cases. (**A**) Focal, only single oocytes are present within field of view. (**B**) Diffuse distribution, more than one oocyte is present in a field of view and are not closely associated with neighbouring oocytes. (**C**) Cluster distribution with 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 D1 is the most advanced development stage of oocytes within a field of view (score 1–5), D2 is the distribution of oocytes within a field of view (score 1–4), and X is the total number of fields of view examined.

$$OSI = \left[\frac{\Sigma [D_1.D_2]}{X} \right]$$

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 summarised as:

Table 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 are rarely 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	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.

Population prevalence of intersex (ovotestis)

The second level of assessment of intersex (ovotestis) 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). Since 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 necessity of detecting a given prevalence (e.g. 1%, 2%, 5%, etc.) and the confidence level of detecting this prevalence (e.g. 90%, 95%, 99%). Whilst 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 the fact 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, multi-site work in several species (e.g. flounder and dab by Stentiford *et al*., 2003 and 2005, respectively) has demonstrated that intersex is detected at some sites and not at others when this regimen is utilised. 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 utilising apparent prevalence in the population, and an indicator for severity in affected individuals.

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

Whilst the link between the formation of intersex (ovotestis) and exposure to anthropogenic contaminants considered to be 'endocrine disrupters' 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 estrogens 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 utilised in monitoring programmes in the OSPAR region, there is a clear historical link between those sites where anthropogenic endocrine disrupters, direct biomarkers of endocrine disruption (e.g. 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 with 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 multi-year 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, since 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.

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 the fact 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, multi-site 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 utilised. 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 utilising 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 due to genetic abnormalities or other causes.

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. Since intersex prevalence is likely to be negligible in non-impacted 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 impacted 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).

Reference

Allen, Y., Scott, A.P., Matthiessen, P., Haworth, S., Thain, J.E., Feist, S.W. 1999a. Survey of estrogenic activity in United Kingdom estuarine and coastal waters and its effect on gonadal development of the flounder *Platichthys flesus*. Environ. Toxicol. Chem. 18, 1791–1800.

Allen, Y., Matthiessen, P., Scott, A.P., Haworth, S., Feist, S.W., Thain, J.E. 1999b. The extent of oestrogenic contamination in the UK estuarine and marine environments—further surveys of flounder. Sci. Total Environ. 233, 5–20.

Amaoka, K., Nishikawa, S., Tanaka, N. 1974. Sexual dimorphism and an abnormal intersexual specimen in the botid flounder *Bothus pantherinus*. Jpn. J. Ichthyol. 21, 16–20.

Bateman, K.S., Stentiford, G.D., Feist, S.W. 2004. A ranking system for the evaluation of intersex condition in European flounder (*Platichthys flesus*). Environ. Toxicol. Chem. 23, 2831–2836.

Blachuta, J., Witkowski, A., Kokurewicz, B. 1991. An hermaphrodite grayling, *Thymallus thymallus* (L.) from the Nysa Klodzka river (Lower Silesia, Poland). J. Fish. Biol. 38, 955–957.

Colborn, T., Dumanoski, D., Peterson Myers, J. 1996. Our stolen future. Abacus, London.

De Metrio, G., Corriero, A., Desantis, S., Zubani, D. *et al*. 2003. Evidence of a high percentage of intersex in the Mediterranean swordfish (*Xiphias gladius* L.). Mar. Pollut. Bull. 46, 358–361.

Devlin, R.H., Nagahama, Y. 2002. Sex determination and sex differentiation in fish: an overview of genetic, physiological, and environmental influences. Aquaculture 208, 191–364.

Dipper, F. 1987. British sea fishes. Underwater World Publications, London.

Feist, S.W., Lang, T., Stentiford, G.D., Koehler, A. 2004. Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring. ICES Tech. Mar. Environ. Sci. 38, 1–48.

Fossi, M.C., Casini, C., Ancora, S., Moscatelli, A., Ausilli, A., Notarbartolo di Sciara, G. 2001. Do endocrine disrupting chemicals threaten Mediterranean swordfish? Preliminary results of vitellogenin and Zona radiata proteins in *Xiphias gladius*. Mar. Environ. Res. 52, 477–483.

Fry, D.M. 1995. Reproductive effects in birds exposed to pesticides and industrial chemicals. Environ. Health Perspect. 103, 165–171.

Gercken, J., Sordyl, H. 2002. Intersex in feral marine and freshwater fish from north-eastern Germany. Mar. Env. Res. 54, 651–655.

Gimeno, S., Gerritson, A., Bowmer, T., Komen, H. 1996. Feminization of male carp. Nature 384, 221–222.

Gimeno, S., Komen, H., Venderbosch, P.W.M., Bowmer, T. 1997. Disruption of sexual differentiation in genetic male common carp (*Cyprinus carpio*) exposed to an alkylphenol during different life stages. Environ. Sci. Technol. 31, 2884–2890.

Hashimoto, S., Bessho, H., Nakamura, M., Iguchi, T., Fujita, K. 2000. Elevated serum vitellogenin levels and gonadal abnormalities in wild male flounder (*Pleuronectes yokohamae*) from Tokyo Bay, Japan. Mar. Environ. Res. 49, 37–53.

Jafri, S.I.H., Ensor, D.M. 1979. Occurrence of an intersex condition in the roach *Rutilus rutilus* L. J. Fish Biol. 14, 547–549.

Jobling, S., Nolan, M., Tyler, C.R., Brightly, G., Sumpter, J.P. 1998. Widespread sexual disruption in wild fish. Environ. Sci. Tech. 32, 2498–2506.

Jobling, S., Burn, R.W., Thorpe, K., Williams, R., Tyler, C. 2009. Statistical Modelling Suggests That Anti-Androgens in Wastewater Treatment Works Effluents are Contributing Causes of Widespread Sexual

Disruption in Fish Living in English Rivers. Environmental Health Perspectives. doi: 10.1289/ehp.0800197.

Kavanagh, R.J., Balch, G.C., Kiparissis, Y., Niimi, A.J., Metcalfe, C.D. 2002. Gonadal intersex in white perch (*Morone Americana*) from the Lower Great Lakes region. In: Conference on Great Lakes Research, Winnipeg, Canada, June 2002. International Association for Great Lakes Research, Ann Arbor, MI, p 64.

Matthiessen, P., Allen, Y., Bignell, J., Craft, J. *et al*. 2000. Studies of endocrine disruption in marine fish—progress with the EDMAR programme. ICES, Copenhagen.

Martin-Skilton, R., Lavado, R., Thibaut, R., Minier, C., Porte, C. 2006. Evidence of endocrine alteration in the red mullet, *Mullus barbatus* from the NW Mediterranean. Env. Poll. 141, 60–68.

Mikaelian, I., De Lafontaine, Y., Harshbarger, J.C., Lee, L.L.J., Martineau, D. 2002. Health of lake whitefish (*Coregonus clupeaformis*) with elevated tissue levels of environmental contaminants. Environ. Toxicol. Chem. 21, 532–541.

Minier, C., Caltot, G., Leboulanger, F., Hill, E.M. 2000. An investigation of the incidence of intersex fish in Seine-Maritime and Sussex regions. Analusis 28, 801–806.

Peters, L.D., Doyotte, A., Mitchelmore, C.L., McEvoy, J., Livingstone, D.R. 2001. Seasonal variation and estradiol-dependent elevation of Thames estuary eel *Anguilla anguilla* plasma vitellogenin and comparisons with other United Kingdom estuaries. Sci. Total Environ. 279, 137–150.

Purdom, C.E., Hardiman, P.A., Bye, V.J., Eno, N.C., Tyler, C., Sumpter, J.P. 1994. Estrogenic effects of effluent from sewage treatment works. Chem. Ecol. 8, 275–285.

Scott, A.P., Sanders, M., Stentiford, G.D. Reese, R.A., Katsiadaki, I. 2007. Evidence for oestrogenic endocrine disruption in an offshore flatfish, the dab (*Limanda limanda* L.). Mar. Env. Res. 64, 128–148.

Sharpe, R.M. 1997. Do males rely on female hormones? Nature 390, 447–448.

Simon, R.C., Schill, W.B. 1984. Tables of sample size requirements for detection of fish infected by pathogens: three confidence levels for different infection prevalence and various population sizes. J. Fish Dis. 7, 515–520.

Slooff, W., Kloowijk-Vandijk, E. 1982. Hermaphroditism in the bream, *Abramis brama* (L.). J. Fish Dis. 5, 79–81.

Stentiford, G.D., Feist, S.W. 2005. First case of intersex (ovotestis) in the flatfish species, dab (*Limanda limanda*): Dogger Bank, North Sea. Mar. Ecol. Prog. Ser. 301, 307–310.

Stentiford, G.D., Longshaw, M., Lyons, B.P., Jones, G., Green, M., Feist, S.W. 2003. Histopathological biomarkers in estuarine fish species for the assessment of biological effects of contaminants. Mar. Environ. Res. 55, 137–159.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Feist, S.W. 2009. Site-specific disease profiles in fish and their use in environmental monitoring. Mar. Ecol. Prog. Ser. 381, 1–15.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Thain, J.E., Feist, S.W. 2010. Age at onset of fish diseases: application to assessment of marine ecological health status. Mar. Ecol. Prog. Ser. 411, 215-230.

van Earle, R., Nolan, M., Jobling, S., Christiansen, L.B., Sumpter, J.P., Tyler, C.R. 2001. Sexual disruption in a second species of wild cyprinid fish (the gudgeon, *Gobio gobio*) in United Kingdom freshwaters. Environ. Toxicol. Chem. 20, 2841–2847.

Vigano, L., Arillo, A., Bottero, S., Massari, A., Mandich, A. 2001. First observation of intersex cyprinids in the Po River (Italy). Sci. Total Environ. 269, 189–194.

Chapter 8: *In vitro* DR-Luc/DR-CALUX® bioassay for screening of dioxin-like compounds in marine and estuarine sediments

Executive summary

Applicability across the OSPAR maritime area. The *in vitro* DR-Luc assay (also called DR-CALUX®, a trademark of BDS, NL, 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), dibenzo-furans (PCDFs) and chlorinated biphenyls (PCBs). The DR-Luc assay is available for immediate deployment within the OSPAR JAMP 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 organised 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 OC content and particle size.

Thresholds and assessment tools. Three assessment classes were derived for DR-Luc based on silica clean-up / 24 h exposure; a background response <10 pg TEQ g^{-1} dry wt; an elevated response (warning level) of >10–<40 pg TEQ g^{-1} dry wt and; a high and cause for concern response of >40 pg TEQ g^{-1} dry wt.

Synergism between CEMP/MSFD and WFD. The DR-Luc bioassay can be immediately applied in offshore and coastal sediments and is equally suitable for estuarine and fresh water sediments (see further also Background Document on water *in vivo* bioassays). As such, the use of DR-Luc can play a role in linking the MSFD with the WFD.

Background

Dioxin levels in the marine environment have declined significantly in the past two decades due to 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 Commission, 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 upon wildlife and human health due to their resistance 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 receptor (AhR)-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 Commission, 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,

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 aryl hydrocarbon (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 ten of the 135 PCDFs are thought to have dioxin-like toxicity (Aarts and Palmer, 2002). For PCBs, only twelve 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.

DR-Luc as bioassay for dioxin-like compounds

The DR-Luc is a reporter gene assay that has been developed by Wageningen University (Aarts *et al*., 1995; Murk *et al*., 1996) and is distributed as DR-CALUX® by Bio Detection System (BDS, NL). 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 with the dioxin concentration. The mode of action of Ah receptor- mediated action is illustrated and further explained in Figure 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.

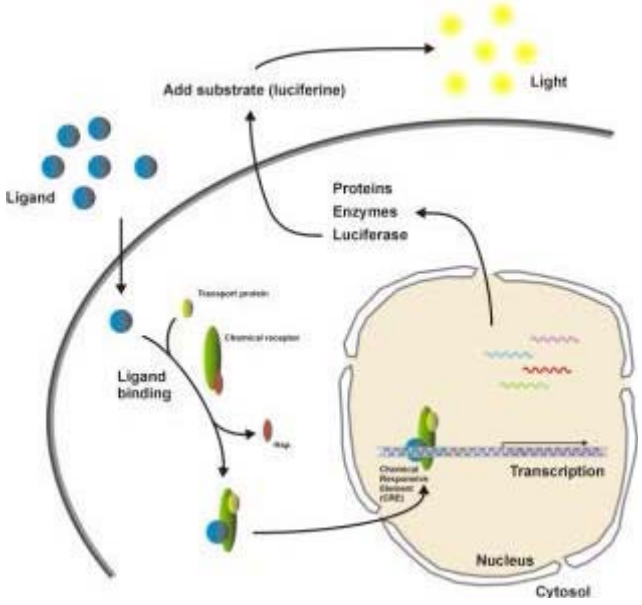

Figure 1 Activation of the Ah-receptor mediated luciferase gene in the DR-Luc bioassay (figure by RIKZ/BDS, 2006). Following activation of the receptor, the ligand–Ah receptor complex translocates to the

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 P4501A1). 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 usually expressed as toxic equivalent quotient (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 basis of concentrations of individual congeners as determined by HRGCMS (see OSPAR Commission, 2007).

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 fresh water 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 recognised within Europe to be an efficient way to assess sediment quality (e.g Hurst *et al*., 2004; Stronkhorst *et al*., 2003; Houtman *et al*., 2004, 2006; 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 national level by several countries (ICES, 2010). 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 SW North Sea) that could be linked with the occurrence of larger PAHs (4–6 rings) (Klamer *et al*., 2005).

From the above studies, it was concluded that the method could be useful as 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 also would have been below. Good correlations were usually observed between DR-Luc/CALUX bioassay results obtained on marine biological matrices and results obtained from 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 of 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.

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 of 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 Effect-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 HRGCMS 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 *et al*., 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 (Schipper *et al*., 2011 submitted).

Synergism between CEMP, MSFD and WFD

Though *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 *et al*., 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.

Thresholds and assessment tools

Three assessment classes were derived for DR-Luc based on silica clean-up / 24 h exposure; a background response <10 pg TEQ g^{-1} dry wt; an elevated response (warning level) of (>10–<40 pg TEQ g^{-1} dry wt and; 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 will become available.

Derivation of AC 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 1). These criteria are "screening levels" which, if exceeded, trigger further investigation at a particular site. Exceeding a screening level does not immediately imply that there is 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 maximum found at locations assumed to be 'clean'. For example, DR-CALUX measurements showed in Dutch surface sediments (Stronkhorst *et al*., 2002; Klamer *et al*., 2005) 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} dw, and on average 24 pg TEQ g^{-1} dw (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} dw, (Klamer *et al*., 2005) and 10–42 pg TEQ g^{-1} dw 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 warning level of >10–< 40 pg TEQ g^{-1} dry wt.

Table 1. International dioxin guidelines (TCDD TEQ) in sediments (dry weight basis).

Country	Maximum allowable Concentration-dry weight basis	Comments	Reference
Vietnam	150 pg/g TEQ	Dioxin heavily contaminated sites (sediments)	Hatfield consultants, 2009
USA	2.5 pg/g TEQ	Protection level	Thain et al., 2006
Canada	4 pg/g TEQ	Protection of ecological receptors	AEA Technology, 1999
Germany	5-10 pg/g TEQ	Protection of human receptors	AEA Technology, 1999
The Netherlands	50 pg/g TEQ	Target value	Stronkhorst, 2002

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. Quality assurance 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 estrogenic 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.

References

Aarts, J.M.M.J.G., Denison, M.S., Cox, M.A., Schalk, C.M.A, Garrison, P.A., Tullis, K., de Haan, L.H.J., Brouwer, A. 1995. Species specific antagonism of Ah receptor action by 2,2',5,5' tetrachloro- and 2,2',3,3',4,4'-hexachlorobiphenyl., Eur. J. Pharm Env. Tox. Section 293:463–474.

Aarts, J.M.M.J.G. and Palmer, R. 2002. Established and deduced aromatic hydrocarbon (Ah) receptor (AhR) agonists and their relative potency. Wageningen University, Department of Agrotechnology and Food Sciences, Wageningen.

AEA Technology. 1999. Compilation of EU dioxin exposure and health data, task 7 - Ecotoxicology. Produced for: European Commission DG Environment and UK Dept. of the Environment, Transport and the Regions (DETR). October. Available online: AEA Technology. 1999. Compilation of EU Dioxin Exposure and Health Data. Prepared for the European Commission DG Environment. Oxfordshire, England.

Aylward, L., Kirman, C., Cher, D., Hays, S. 2003. Analysis of Dioxin Cancer Threshold. Journal article by Environmental Health Perspectives 111: 1145–1147.

Aylward, L.L. and Hays, S.M. 2002. Temporal trends in human TCDD body burden: Decreases over three decades and implications for exposure levels. Journal of Exposure Analysis and Environmental Epidemiology 12: 319–28.

Besselink, H., Jonas, A., Pijnappels, M., Swinkels, A., Fjellanger, K. and Brouwer, A. 2003. Comparison of DR CALUX® and HRGCMS-derived TEQs: introduction of conversion factors. *Organohalogen Compounds*, 60, pp 203–206.

Besselink, H.T., Schipper, C., Klamer, H., Leonards, P., Verhaar, H., Felzel, E.C., Murk, A.J., Thain, J., Hosoe, K., Schoeters, G., Legler, J. and Brouwer, A. 2004. Intra- and interlaboratory calibration of the DR-CALUX bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. *Environmental Toxicology and Chemistry, 23*(12), 2781–2789.

Bosveld, A.T.C., Graneder, J., Murk, A.J., Brouwer, A., Kampen, M. van, Evers, E.H.G. and Berg, M. van den. 1995. Effects of PCBs, PCDDs, and PCDFs in common tern (*Stern hirundo*) breeding in estuarine and coastal colonies in the Netherlands and Belgium. Environ. Toxicol. Chem. 14: 99–115.

Burgess, R.M. 2000. Characterising and identifying toxicants in marine waters: a review of marine Toxicity Identification Evaluations (TIEs). International Journal of Environment and Pollution 13: 2–33.

De Boer, J. and Wells, D.E. 1996. The 1994 QUASIMEME laboratory-performance studies: chlorobiphenyls and organochlorine pesticides in fish and sediment. Mar. Pollut. Bull. 32: 654–666.

Foekema, E.M., Deerenberg, C., Murk, A.J. 2008. Prolonged ELS test with the marine flatfish sole (*Solea solea*) shows delayed toxic effects of previous exposure to PCB 126. Aquat. Toxicol. 90: 197–203.

Hamers, T., Leonards, P.E.G. Legler, J., Vethaak, A.D., Schipper, C.A. 2010. Toxicity Profiling: an effect-based integrative tool for site-specific sediment quality assessment. Integrated Environmental Assessment and Management 6: 761–773.

Hatfield Consultants. 2009. Comprehensive assessment of dioxin contamination in Da Nang airport, Vietnam: environmental levels, human exposure and options for mitigating impact. DANDI-II 1450.3, Hatfield Consultants Parnership.

Heilier, J.F., Nackers, F., Verougstraete, V., Tonglet, R., Lison, D. and Donnez, J. 2005. Increased dioxin-like compounds in the serum of women with peritoneal endometriosis and deep endometriotic (adenomyotic) nodules, Fertil. Steril. 84: 305–312.

Henshel, D.S. 1998. Developmental neurotoxic effects of dioxin and dioxin-like compounds on domestic and wild avian species. Environ Toxicol Chem 17: 88–98.

Hoogenboom, R., Bovee, T., Traag, W., Hoogerbrugge, R., Baumann, B., Portier, L., van de Weg, G., de Vries, J. 2006. The use of the DR CALUX bioassay and indicator polychlorinated biphenyls for screening of elevated levels of dioxins and dioxin-like polychlorinated biphenyls in eel. Mol Nutr Food Res. 50(10): 945–57.

Hoogenboom, R. 2002.The combined use of the CALUX bioassay and the HRGC/HRMS method for the detection of novel dioxin sources and new dioxin-like compounds. Environmental Science and Pollution Research. 9(5): 304–306.

Houtman, C.J., Booij, P., Jover, E., Pascual del Rio, D., Swart, K., Velzen, M. van, Vreuls, R., Legler, J., Brouwer, A. & Lamoree, M.H. 2006. Estrogenic and dioxin-like compounds in sediment from Zierikzee harbour identified with CALUX assay-directed fractionation combined with one and two dimensional chromatography analyses. Chemosphere 65: 2244–2252.

Houtman, C.J., Cenijn, P.H., Hamers, T., Lamoree, M.H., Legler, J., Murk, A.J., Brouwer, A. 2004. Toxicological profiling of sediments using *in vitro* bioassays, with emphasis on endocrine disruption. Environ. Toxicol. Chem. 23:32–40.

Hurst, M.R., Balaam, J., Chan-Man, Y.L., Thain, J.E., Thomas, K.V. 2004. Determination of dioxin and dioxin-like compounds in sediments from UK estuaries using a bio-analytical approach: chemical-activated luciferase expression (CALUX) assay Marine Pollution Bulletin 49: 648–658.

ICES. 2010. Report of the Working Group on biological effects of contaminants. Report 2010.

Klamer, H.J.C., Leonards, P.E.G., Lamoree, M.H., Villerius, L.A., Åkerman, J.E., Bakker, J.F. 2005. A chemical and toxicological profile of Dutch North sea surface sediments. Chemosphere 58: 1579–1587.

Legler, J., Bulder, A.S., De Voogt, P., Leonards, P.E.G., Murk, T.A.J. 2006a. Bioanalysis of estrogenic and dioxin-like activity in field samples, in estrogens and xenooestrogens in the aquatic environment: an integrated approach for field monitoring and effect assessment, (editors; Vethaak D., Schrap M., De Voogt P., SETAC, ISBN-13:978-1-880611-85-2, pp. 121–149, USA.

Legler, J., Brouwer, A. and Lamoree, M.H. 2006b. Estrogenic and dioxin-like compounds in sediment from Zierikzee harbour identified with CALUX assay-directed fractionation combined with one and two dimensional gas chromatography analyses. Chemosphere, 65, 2244–2252.

Leonards, P.E.G., Van Hattum, B. and Leslie, H. 2008. Assessing the risks of persistent organic pollutants to top predators: a review of approaches. Integrated Environmental Assessment and Management, Vol 4, 386–398.

Liem A.K.D. and J.A. van Zorge. 1995. Dioxins and related compounds: Status and regulatory aspects. Environmental Science and Pollution Research 2: 46–56.

Maas, J.L., van de Heuvel-Greve, M. 2005. Opportunities for bio-analysis in WFD chemical monitoring using bioassays. RWS RIZA/RIKZ. Internal report.

Mandal, P.K. 2005. Dioxin: a review of its environmental effects and its aryl hydrocarbon receptor biology Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology 175 (4): 221–230.

Murk, A.J., P.E.G. Leonards, A.S. Bulder, *et al.* 1997. The CALUX (chemical activated luciferase expression) assay adapted and validated for measuring TCDD equivalents in blood plasma. *Environm. Toxicol. & Chem.* 16 (8), 1583–9.

Murk, A.J., Legler, J. Denison, M.S., Giesy, J.P., Van de Guchte, C. and Brouwer, A. 1996. Chemical-activated luciferase gene expression (CALUX): A novel *in vitro* bioassay for Ah receptor active compounds in sediments and pore water. Fund. Appl. Toxicol. 33: 149–160.

Murk, A.J., Leonards, P.E.G., Hattum, B. van, Luit, R., Weiden, M.E.J. van der and Smit, M. 1998. Application of biomarkers for exposure and effect of polyhalogenated aromatic hydrocarbons in naturally exposed European otters (*Lutra lutra*). Environ. Toxicol. Pharm. 6(2): 91–102.

Oris, J. and Klaine. S. 2000. Whole effluent toxicity testing. Environmental Toxicology and Chemistry 1–256.

OSPAR Commission. 2007. Hazardous Substances Series OSPAR Background Document on Dioxins, 56 pp.

Power, E.A. 2004. International Trends in Bioassay Use for Effluent Management. Ecotoxicology 13 (5): 377–398.

Puga, A., Tomlinson, C.R., Xia, Y. 2005. Ah receptor signals cross-talk with multiple developmental pathways. Biochem Pharmacol. 69(2): 199–207.

Rappe, C. 1996. Sources and environmental concentrations of dioxins and related compounds, Pure and Applied Chemistry 68 (9): 1781–1789.

RIKZ. 2006. RIKZ/SPECIE-07; Determination of 2,3,7,8-TCDD Toxic Equivalents (TCDD-TEQs) in sediments with the DR CALUX a bioassay by BDS. RIKZ, Middelburg, The Netherlands.

Sanctorum, H., Windal, I., Hanot, V., Goeyens, L., and Baeyens, W. 2007. Dioxin and dioxin-like activity in sediments of the Belgian Coastal area (Southern North Sea). Archives of Env. Cont. and Tox. 52: 317–325.

Schipper, C.A., Joost, L., George, S.G. , Hansen, P-D., da Silva de Assis, H.C., van der Oost, R., Thain, J.E., Livingstone, D.R., Mitchelmore, C.L., van Schooten, F.J., Ariese, F., Murk, A.J., Grinwis, G.C.M., Klamer, H.J.C., Kater, B.J., Postma J.F., van den Brink, P.J., van der Werf, D.C.B., Vethaak, A.D. 2009. Application of biomarkers in flounder (*Platichthys flesus*) and bioassays in contaminated estuarine sediments, 66, ICES Journal of Marine Science, 66 (10): 2089–2105.

Schipper, C.A., Burger, R.M., Rietjens, I.M.C.M., Murk, A.T. 2010. Application of bioassays in toxicological hazard, risk and impact assessments of dredged sediments. Marine Pollution Bulletin 60: 2026–2042.

Schipper, C.A., Leonards, P.E.G., Klamer, H.J.C., Thomas, K.V., Vethaak, A.D. 2011. Biological effects of contaminants: Receptor H4IIE-Luciferase (DR-LUC) cell bioassay for screening of dioxins and/or dioxin-like compounds in environmental samples, ICES TIMES (submitted).

Schoeters, G., Legler, J., Brouwer, A. 2009. Intra- and interlaboratory calibration of the DR-CALUX® bioassay Bradfield CA. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. Immunology 2009; 127(3):299–311.

Stevens, E.A., Mezrich, J.D., Bradfield, C.A. 2009. The aryl hydrocarbon receptor: a perspective on potential roles in the immune system. Immunology.127(3):299–311.

Stronkhorst, J., Leonards, P., and Murk, A.J. 2002. Using the dioxin receptor-CALUX *in vitro* bioassay to screen marine harbour sediments for compound with a dioxin like-mode of action, Environmental Toxicology and Chemistry 21: 2552–2561.

Stronkhorst, J., Ariese, F., van Hattum, B., Postma, J.F., de Kluijver, M., Den Besten, P.J., Bergman, M.J.N., Daan, R., Murk, A.J. and Vethaak, A.D. 2003. Environmental impact and recovery at two dumping sites for dredged material in the North Sea. Environ. Pollut. 124: 17–31.

Thain, J.E., Hurst, M.R. and Thomas, K.V., 2006. Determination of dioxin-like activity in sediments from the East Shetland basin. Organohal. Cpds. 68, 185-188.

Van den Berg, M., Birnbaum, L., Bosveld, A.T., Brunstrom, B., Cook, P., Feeley, M., *et al*. 1998. Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife Environ. Health Perspect. 106: 775–792.

Van den Brink, P.J. and Kater, B.J. 2006. Chemical and biological evaluation of sediments from the Wadden Sea, The Netherlands, Ecotoxicology 15: 451–460.

Van Loco, J., Van Leeuwen, S.P.J., Roos, P., Carbonnelle, S., de Boer, J., Goeyens, L., Beernaert, H. 2004. The international validation of bio- and chemical- analytical screening methods for dioxins and dioxin-like PCBs: the DIFFERENCE project rounds 1 and 2 TALANTA 63 (5): 1169–1182.

Vos, J.G., Dybing, E., Greim, H.A., Ladefoged, O., Lambré, C., Tarazona, J.V., Brandt, I. and Vethaak, A.D. 2000. Health effects of endocrine-disrupting chemicals on wildlife, with special reference to the European situation. Critical reviews in toxicology 30(1): 71–133.

WFD CIS. 2003. Guidance document No. 3; Analysis of Pressures and Impacts in accordance with the Water Framework Directive. www.circa.eu (April 2009).

Windal, I., van Wouwe, N., Eppe, G., Xhrouet, C., Debacker, V., Baeyens, W., de Pauw, E., Goeyens, L. 2002. Validation and interpretation of CALUX as a tool for the estimation of dioxin-like activity in marine detection of novel dioxin sources and new dioxin-like compounds. Environ Sci & Pollut Res 9: 304–306.

World Health Organization. 2000. Interlaboratory Quality Assessment of Levels of PCBs, PCDDs, and PCDFs in Human Milk and Blood Plasma; fourth round of WHO-coordinated study. WHO, Bilthoven.

Chapter 9: Lysosomal stability as a global health status indicator in biomonitoring

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, and lysosomal membrane stability is a good diagnostic biomarker of individual health status (Allen and Moore, 2004; Bayne and Moore, 1998; Burlando *et al*., 2002; Cajaraville *et al*., 1995, 2000; Dondero *et al*., 2006b; Galloway *et al*., 2002, 2004; Hankard *et al*., 2004; Klionsky and Emr, 2000; Köhler *et al*., 1992, 2002; Lekube *et al*., 2000; Lowe, 1988; Lowe *et al*., 1992, 1995, 2006; Marigomez and Baybay-Villacorta, 2003; Moore, 1976, 1985, 1988, 1990, 2002; Moore *et al*., 2004a; Moore *et al*., 2006a,b,c; Nicholson and Lam, 2005; Svendsen and Weeks, 1995; Svendsen *et al*., 2004; Winston *et al*., 2002). Dysfunction of lysosomal processes has been mechanistically linked with many aspects of pathology associated with toxicity and degenerative diseases (Cuervo, 2004; Köhler, 2004; Köhler *et al*., 2002; 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 capability 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 recently been adopted by UNEP as part of the first tier of techniques for assessing harmful impact in the Mediterranean Pollution programme (MEDPOL Phase IV). Other lysosomal biomarkers including lipofuscin in molluscs (age/stress pignment), and lysosomal neutral lipid (chemically induced lipidosis) in molluscs and fish have been adopted as part of the second tier assessment methods (Krishnakumar *et al*., 1994; Moore, 1988; 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*., 1999 a, b; Köhler *et al*., 2002; Köhler, 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 (Allen and Moore, 2004; Kirchin *et al*., 1992; Köhler *et al*., 2002; Moore *et al*., 2004a, 2006a; Nicholson and Lam, 2005; Svendsen and Weeks, 1995; Svendsen *et al*., 2004).

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 (Köhler *et al*. 2002; Moore, 1988; Moore *et al*., 2006a, b; Viarengo *et al*., 1985a). Metals such as copper, cadmium and mercury will also induce lysosomal destabilisation in mussels (Viarengo *et al*., 1981, 1985a, b), and if oxyradicals are generated then lipofuscinosis can also occur (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 (Dailianis *et al*., 2003; Kalpaxis *et al*., 2004; Krishnakumar *et al*., 1994; Moore *et al*., 2004a, b, 2006a; Regoli, 2000; Ringwood *et al*., 2004). In fish liver, lysosomal membrane stability is strongly correlated with a suppression of the activity of macrophage aggregates (Broeg, 2003; Broeg *et al*., 2005), lipidosis and lipofuscinosis (Broeg *et al*., 1999 a,b; Broeg *et al*., in preparation; Köhler , 2004).

Lysosomal stability and other lysosomal biomarkers such as lipofuscin are strongly correlated with

mussel tissue concentration of PAHs, which are ubiquitous contaminants (Cajaraville *et al*., 2000; Krishnakumar *et al*., 1994; Moore, 1990; Moore *et al*., 2006a, b, c; Viarengo *et al*., 1992), 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; Schiedek *et al*., 2006, Baršiene *et al*., 2006; Sturve *et al*., 2005), single pollution events and accidents (Einsporn *et al*. 2005; Broeg *et al*., 2002, Nicholson and Lam, 2005) and also serves for the discovery of new "Hot Spots" of pollution (Bressling, 2006; Moore *et al*., 1997, 1998a,b; 2004).

A conceptual mechanistic model has been developed linking lysosomal damage and autophagic dysfunction with injury to cells, tissues and the whole animal; and the complementary use of cell-based bioenergetic computational model of molluscan hepatopancreatic cells that simulates lysosomal and cellular reactions to pollutants has also been demonstrated (Allen and McVeigh, 2004; Lowe, 1988; 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 (Allen and Moore, 2004; Clarke, 1999; Dagnino *et al*., 2007; Dondero *et al*., 2006a; Lowe, 1988; Moore, 1988; Moore *et al*., 2006a). 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 ariabili 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 multi-dimensional health status space are multiple contaminant and biomarker data, environmental ariability, 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).

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; Moore *et al*., 2007). Processing for neutral red retention (NRR) in samples of molluscs adapted to low salinity environments 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 *et al*., 1978). In general, this period should be avoided anyway for sampling purposes, as 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).

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), and 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).

Quality Assurance

Intercalibration exercises for lysosomal stability techniques have been carried out in the ICES/UNESCO-IOC-GEEP Bremerhaven Research Workshop and UNEP-MEDPOL programme, and for the neutral red retention 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 inter-laboratory 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 neutral red retention techniques have been performed in fish liver (ICES-IOC Bremerhaven Workshop, 1990) and in mussels experimentally exposed to PAHs (Lowe *et al*., 1995).

Background Responses and Assessment Criteria

Health status thresholds for NRR and cytochemical methods for lysosomal stability have been determined from data based on numerous studies (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 minutes; stressed but compensating if <20 but ≥10 minutes and severely stressed and probably exhibiting pathology if <10 minutes (Moore *et al*., 2006a). Similarly for the NRR method, animals are considered to be healthy if NRR is ≥120 minutes; stressed but compensating if <120 but ≥ 50 minutes and severely stressed and probably exhibiting pathology if <50 minutes (Moore *et al*., 2006a).

References

Allen, J.I. and Moore, M.N. 2004. Environmental prognostics: is the current use of biomarkers appropriate for environmental risk evaluation. Mar. Environ. Res., 58: 227- 232.

Allen, J.I. and McVeigh, A. 2004. Towards computational models of cells for environmental toxicology. J. Mol. Histol., 35: 697-706.

Bayne, C.J. and Moore, M.N. 1998. Non-lymphoid immunologic defenses in aquatic invertebrates and their value as indicators of aquatic pollution. In: EcoToxicology: Responses, Biomarkers and Risk Assessment (Ed. J.T. Zelikoff), pp. 243 261. Published for OECD by SOS Publications, Fair Haven, New Jersey.

Baršiene, J., Lehtonen, K. K., Koehler, A., Broeg, K., Vuorinen, P. J., Lang, T., Pempkowiak, J., Balk, L., Syvokiene, J., Dedonyte, V., Rybakovas, A., Repecka, R., Vuontisjärvi, H. and Kopecka, J. 2006. Biomarker responses in mussel (*Mytilus edulis*) and flounder (Platichthys flesus) in the Klaipeda-Butinge area (Baltic Sea). Mar. Poll. Bull., 53: 422-436.

Bressling, J. 2006. Natural and toxic stress indicator in blue mussel *Mytilus edulis* along an offshore-inshore gradient (Weser estuary). Master's Thesis, University of Oldenburg, pp122.

Broeg, K., Köhler, A. and von Westernhagen, H. 2002. Disorder and recovery of environmental health monitoring by means of lysosomal stability in liver of European flounder (*Platichthys flexus L*.). Mar. Environ. Res., 54: 569-573.

Broeg, K., Zander, S., Diamant, A., Körting, W., Krüner, G., Paperna, I. and von Westernhagen, H. 1999a. The use of fish metabolic, pathological and parasitological indices in pollution monitoring. I. North Sea. Helgoland Mar. Res., 53: 171-194.

Broeg, K., Zander, S., Diamant, A., Körting, W., Krüner, G., Paperna, I., and von Westernhagen, H. 1999b. The use of fish metabolic, pathological and parasitological indices in pollution monitoring I. Red Sea and Mediterranean Sea. Helgoland Mar. Res., 53: 195-208.

Broeg, K., Kaiser, W., Bahns, S. and Köhler, A. The liver of wrasse - morphology and function as a mirror of point source chemical impact. In preparation.

Burlando, B., Evangelisti, V., Dondero, F., Pons, G., Camakaris, J. and Viarengo, A. 2002. Occurrence of Cu-ATPase in Dictyostelium: possible role in resistance to copper. Biochem. Biophys. Res. Comm., 291: 476-483.

Cajaraville, M.P., Bebianno, M.J., Blasco, J., Porte, C., Sarasquete, C. and Viarengo, A. 2000. The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci. Tot. Environ., 247: 295-311.

Cajaraville, M.P., Abascal, I., Etxeberria, M. and Marigómez, I. 1995. Lysosomes as cellular markers of environmental pollution: time- and dose-dependant responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Environ. Toxicol. Water Qual., 10: 1-8.

Clarke, K.R. 1999. Non-metric multivariate analysis in community-level ecotoxicology. Environ. Toxicol. Chem., 18: 117-127.

Cuervo, A.M. 2004. Autophagy: in sickness and in health. TRENDS in Cell Biology, 14, 70-77.

Dagnino, A., Allen, J.I., Moore, M., Canesi, L. and Viarengo A. 2007. Integration of biomarker data into an organism health index: development of an expert system and its validation with field and laboratory data in mussels. Biomarkers, In press.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kaloyianni, M. and Dimitriadis, V.K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Mar. Environ. Res., 56: 443-470.

Da Ros, L., Meneghetti, F. and Nasci, C. 2002. Field application of lysosomal destabilisation indices in the mussel *Mytilus galloprovincialis*: biomonitoring and transplantation in the Lagoon of Venice (north-east Italy), Mar. Environ. Res., 54: 817-822.

Dondero, F., Dagnino, A., Jonsson, H., Capri, F., Gastaldi, L. and Viarengo, A. 2006a. Assessing the occurrence of a stress syndrome in mussels (*Mytilus edulis*) using a combined biomarker/gene expression approach. Aquatic Toxicol., 78S, S13-S24.

Dondero, F., Jonsson, H., Rebelo, M., Pesce, G., Berti, E., Pons, G. and Viarengo, A. 2006b. Cellular responses to environmental contaminants in amoebic cells of the slime mould *Dictyostelium discoideum*. Comp. Biochem. Physiol., 143C: 150-157.

Einsporn, S., Broeg, K. and Köhler, A. 2005. The Elbe flood 2002-toxic effects of transported contaminants in flatfish and mussel of the Wadden Sea. Mar. Pollut. Bull., 50: 423-429.

Galloway, T.S., Sanger, R.C., Smith, K.L., Fillmann, G., Readman, J.W., Ford, T.E. and Depledge, M.H. 2002. Rapid assessment of marine pollution using multiple biomarkers and chemical immunoassays. Environ.Sci.Technol., 2002, 36: 2219-2226.

Galloway, T.S., Brown, R.J., Browne, M.A., Dissanayake, A., Lowe, D., Jones, M.B. and Depledge, M.H. 2004. A multibiomarker approach to environmental assessment. Environ. Sci. Technol., 38: 1723- 731.

Hankard, P.K., Svendsen, C., Wright, J., Wienberg, C., Fishwick, S.K., Spurgeon, D.J. and Weeks, J.M. 2004. Biological assessment of contaminated land using earthworm biomarkers in support of chemical analysis. Sci. Total Environ. 330: 9-20.

Kalpaxis, D.L., Theos, C., Xaplanteri, M.A., Dinos, G.P., Catsiki, A.V. and Leotsinidis, M. 2004. Biomonitoring of Gulf of Patras, N. Peloponnesus, Greece. Application of a biomarker suite including

evaluation of translation efficiency in *Mytilus galloprovincialis* cells. Environ. Res., 94: 211-220.

Kirchin, M.A., Moore, M.N., Dean, R.T. and Winston, G.W. 1992. The role of oxyradicals in intracellular proteolysis and toxicity in mussels. Mar. Environ. Res., 34: 315-320.

Klionsky, D.J. and Emr, S.D. 2000. Autophagy as a regulated pathway of cellular degradation. Science, 290: 1717-1721.

Köhler, A., 1990. Identification of contaminant-induced cellular and subcellular lesions in the liver of flounder (*Platichthys flesus L*.) caught at differently polluted estuaries. Aquat. Toxicol. 16: 271- 294.

Köhler, A. 1991. Lysosomal perturbations in fish liver as indicator for toxic effects of environmental pollution. Comp. Biochem. Physiol., 100C: 123-127.

Köhler, A. and Pluta, H.J., 1995. Lysosomal injury and MFO activity in the liver of flounder (*Platichthys flesus L*.) in relation to histopathology of hepatic degeneration and carcinogenesis, Mar. Environ. Res., 39: 255-260.

Köhler, A., Alpermann, T., Lauritzen, B. and Van Noorden, C.J.F. 2004. Clonal xenobiotic resistance during pollution-induced toxic injury and hepatocellular carcinogenesis in liver of female flounder. Acta Histochem., 106: 155-170.

Köhler, A., Deisemann, H. and Lauritzen, B. 1992. Ultrastructural and cytochemical indices of toxic injury in dab liver. Mar. Ecol. Prog. Ser., 91: 141-153.

Köhler, A., Wahl, E. and Söffker, K. 2002. Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish (*Platichthys flesus (L)*). Environ. Toxicol. Chem., 21: 2434-2444.

Krishnakumar, P.K., Casillas, E. and Varanasi, U. 1994. Effect of environmental contaminants on the health of *Mytilus edulis* from Puget Sound, Washington, USA. I. Cytochemical measures of lysosomal responses in the digestive cells using automatic image analysis. Mar. Ecol. Prog. Ser., 106: 249-261.

Lehtonen, K., Schiedek, D., Koehler, A., Lang, T., Vuorrinen, P.J., Foerlin, L., Baršiene, J., Pempkowski, A.J., Gercken, J. 2006. The Beep Project in the Baltic Sea: Overview of results and outline for a regional biological effects monitoring strategy. Mar. Poll. Bull., 53: 523-537.

Lekube, X., Cajaraville, M.P. and Marigomez, I. 2000. Use of polyclonal antibodies for the detection of changes induced by cadmium in lysosomes of aquatic organisms. Sci. Total Environ., 247: 201-212.

Lowe, D.M. 1988. Alterations in the cellular structure of *Mytilus edulis* resulting from exposure to environmental contaminants under field and experimental conditions. Mar. Ecol. Prog. Ser., 46: 91-100.

Lowe, D.M., Fossato, V.U. and Depledge, M.H. 1995. Contaminant induced lysosomal membrane damage in blood cells of mussels *M. galloprovincialis* from the Venice Lagoon: an *in vitro* study. Mar. Ecol. Prog. Ser., 129: 189-196.

Lowe, D.M., Moore, M.N. and Clarke, K.R. 1981. Effects of oil on digestive cells in mussels: quantitative alterations in cellular and lysosomal structure. Aquatic Toxicol., 1: 213-226.

Lowe, D.M., Moore, M.N. and Evans, B.M. 1992. Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar. Ecol. Prog. Ser., 91: 135-140.

Lowe, D.M., Moore, M.N. and Readman, J.W. 2006. Pathological reactions and recovery of hepatopancreatic digestive cells from the marine snail Littorina littorea following exposure to a polycyclic aromatic hydrocarbon. Mar. Environ. Res., 61: 457-470.

McVeigh, A., Allen, J.I., Moore, M.N., Dyke, P. and Noble, D. 2004. A carbon and nitrogen flux model of mussel digestive gland epithelial cells and their simulated response to pollutants. Mar. Environ. Res., 58: 821-827.

Marigomez, I. and Baybay-Villacorta, L. 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquatic Toxicol., 64: 235-257.

Moore, M.N. 1976. Cytochemical demonstration of latency of lysosomal hydrolases in digestive cells of the common mussel, *Mytilus edulis* and changes induced by thermal stress. Cell Tissue Res., 175: 279 287.

Moore, M.N. 1985. Cellular responses to pollutants. Mar. Poll. Bull., 16: 134-139.

Moore, M.N. 1988. Cytochemical responses of the lysosomal system and NADPH-ferrihemoprotein reductase in molluscs to environmental and experimental exposure to xenobiotics. Mar. Ecol. Prog. Ser., 46: 81-89.

Moore, M.N. 1990. Lysosomal cytochemistry in marine environmental monitoring. Histochem. J., 22: 187 191.

Moore, M.N. 2002. Biocomplexity: the post-genome challenge in ecotoxicology. Aquatic Toxicol., 59: 1-15.

Moore, M.N., Allen, J.I. and McVeigh, A. 2006a. Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar. Environ. Res. In press.

Moore, M.N., Allen, J.I., McVeigh, A. and Shaw, J. 2006c. Lysosomal and autophagic reactions as predictive indicators of environmental impact in aquatic animals. Autophagy, 2: 217-220.

Moore, M.N., Allen, J.I. and Somerfield, P.J. 2006b. Autophagy: role in surviving environmental stress. Marine Environmental Research, 62 Suppl. 1: S420-S425.

Moore, M.N., Depledge, M.H., Readman, J.W. and Leonard, P. 2004a. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutation Res., 552: 247-268.

Moore, M.N., Koehn R.K. and Bayne, B.L. 1980. Leucine aminopeptidase (aminopeptidase-1), N-acetyl-β-hexosaminidase and lysosomes in the mussel *Mytilus edulis L*., in response to salinity changes. J. Exp. Zool., 214: 239-249.

Moore, M.N., Lowe, D. and Köhler, A. 2004b. Measuring lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences, No. 36. ICES, Copenhagen, 31 p.

Moore, M. N., Lowe, D. M., Wedderburn, R. J., Wade, T., Balashov. G., Büyükgüngör, H., Daurova, Y., Denga, Y., Kostylev, E., Mihnea, P., Moncheva, S.Tabagari, S., Ciocan, C., Özkoc, H., Depledge, M. H. 1998a. International Mussel Watch (UNESCO/IOC) in the Black Sea: a pilot study for biological effects and contaminant residues. In: Environmental degradation of the Black Sea, (Eds. S. Beşiktepe *et al*.), pp. 273 – 289. Kluwer Academic Publishers, Amsterdam.

Moore, M.N., Topping, G., Minhea, P.-E., Kiryanov, S. and Mikhailov, V. 1998b. Design of a monitoring programme for the Black Sea: contaminant levels and biological effects. In: Black Sea Pollution Assessment (Eds. Mee, L. and Topping,G.), 293-299. UN Publications, New York.

Moore, M.N., Viarengo, A., Donkin, P. and Hawkins, A.J.S. 2007. Autophagic and lysosomal reactions to stress in the hepatopancreas of blue mussels. Aquatic Toxicology, In press.

Moore, M.N., Wade, T.L., Lowe, D.M., Wedderburn, R.J., Balashov, G., Büyükgüngör, H., Daurova, Y., Denga, Y., Kostylev, E., Mihnea, P., Moncheva, S., Tabagari, S., Ciocan, C., Özkoc, H. and Depledge, M.H. 1997. The UNESCO/IOC "Black Sea Mussel Watch Pilot Study" : biological effects and contaminant residues. In: Black Sea Pollution Assessment (Eds. Mee, L. and Topping,G.), pp. 279-292. UN Publications, New York.

Nicholson, S. and Lam, P.K.S. 2005. Pollution monitoring in South-East Asia using biomarkers in the mytilid mussel *Perna viridis* (Mytilidae: Bivalvia). Environ. Internat., 31: 121-132.

Pisoni, M., Cogotzi, L., Frigeri, A., Corsi, I., Bonacci, S., Iacocca, A., Lancini, L., Mastrototaro, F., Focardi, S., Svelto *et al*. 2004. DNA adducts, benzo(a)pyrene monooxygenase activity, and lysosomal membrane stability in *Mytilus galloprovincialis* from different areas in Taranto coastal waters (Italy). Environ. Res., 96: 163-175.

Regoli, F. 2000. Total oxyradical scavenging capacity (TOSC) in polluted and translocated mussels: a predictive biomarker of oxidative stress. Aquatic Toxicol.: 50, 351-361.

Ringwood, A.H., Hoguet, J., Keppler, C. and Gielazyn, M. 2004. Linkages between cellular biomarker responses and reproductive success in oysters, *Crassostrea virginica*. Mar Environ. Res., 58: 151-155.

Schiedek, D., Broeg, K., Baršiene, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P. J., Köhler, A., Balk, L., Schneider, R. 2006. Biomarker responses and indication of contaminant effects in blue mussel (*Mytilus edulis*) and eelpout (*Zoarces viviparus*) from the western Baltic Sea. Mar. Poll. Bull., 53: 387- 405.

Sturve, J., Balk, L., Berglund, A., Broeg, K., Böhmert, B., Koehler, A., Massey, S., Parkkonen, J., Savva, D., Stephensen, E., Förlin, L. 2005. Effects of dredging in Goteborg harbour assessed by biomarkers in eelpout (*Zoarces viviparus*). Environ. Toxicol. Chem., 24: 1951-1961.

Svendsen, C. and Weeks, J.M. 1995. The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). Mar. Poll. Bull. : 31, 139- 42.

Svendsen, C., Spurgeon, D.J., Hankard, P.K. and Weeks, J.M. 2004. A review of lysosomal membrane stability measured by neutral red retention: is it a workable earthworm biomarker? Ecotox. Environ. Safety, 57: 20-29.

Viarengo, A., Lafaurie, M., Gabrielides, G.P., Fabbri, R., Marro, A. and Romeo, M. 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MEDPOL biomonitoring program. Mar. Environ. Res., 49: 1-18.

Viarengo, A., Moore, M.N., Mancinelli, G., Mazzucotelli, A. and Pipe, R.K. 1985a. Significance of metallothioneins and lysosomes in cadmium toxicity and homeostasis in the digestive gland cells of mussels exposed to the metal in the presence and absence of phenanthrene. Mar. Environ. Res., 17, 184-187.

Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Accomando, R. 1992. A simple procedure for evaluating the protein degradation rate in mussel (*Mytilus galloprovincialis Lam*.) tissues and its application in a study of phenanthrene effects on protein catabolism. Comp. Biochem. and Physiol., 103B: 27- 32.

Viarengo, A., Moore, M.N., Pertica, M., Mancinelli, G., Zanicchi, G. and Pipe, R.K. 1985b. Detoxification of copper in the cells of the digestive gland of mussel: the role of lysosomes and thioneins. Sci. Tot. Environ., 44: 135-145.

Viarengo, A., Zanicchi, G., Moore, M.N. and Orunesu, M. 1981. Accumulation and detoxication of copper by the mussel *Mytilus galloprovincialis*. Aquatic Toxicol., 1: 147-157.

Winston, G.W., Adams, S.M., Benson, W.H., Gray, L.E., Matthews, H.S., Moore, M.N., and Safe, S. 2002. Biological bases of similarities and differences. In: Interconnections between Human Health and Ecological Integrity, (Eds. R.T. Di Giulio and W.B. Benson), pp. 43-65. Society of Environmental Toxicology and Chemistry (SETAC), Pensacola, Florida.

Chapter 10: Metallothionein (MT) in blue mussels (*Mytilus edulis, Mytilus galloprovincialis*)

Introduction

Metallothionein (MT) is a low-molecular-weight, cystein-rich protein, metal-binding protein that is 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:

i) regulating cellular processes requiring Zn and/or Cu; and

ii) 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 due to the large number of SH-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). Since 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 *Mytilus 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).

Methods for quantification

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

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

iv) metal-substitution; and

v) the spectrophotometric sulphydryl 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 sulphydryl-groups (SH-groups) in MT and its small size. There has been an international intercalibration of method (iii) through MEDPOL (Viarengo *et al*., 2000) and of fish MT using all three methods within the BEQUALM framework (Hylland, unpublished). 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 e.g. Cd. Method (iii) gives different results to the other methods, resulting in either over- or underestimation. None of the methods are able to separate 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 *et al*., 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 anyway 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 about 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.

Concentrations in reference areas

A range of studies have quantified MT using differential pulse polarography in whole mussel, hepatopancreas and/or gill in *M. edulis* (Table 1) or *M. galloprovincialis* (Table 2). A smaller number of studies have been using the sulphydryl method (Table 3). Early analyses using metal-substitution assays will have underestimated MT and have not been included in this overview.

Table 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 0.8 (water content; see e.g. Williams, 1970) and from protein-standardised values using a factor 0.08 (assuming 2/3 cytosolic protein and a protein content of 60% of dry wt; Dare & Edwards, 1975).

tissue	original value	factor	MT (µg/g ww)	Reference
Whole animal	2.43	0.2	0.49	Bebianno and Langston (1989)
	2.75	0.2	0.55	Bebianno and Langston (1991)
	0.55	1	0.55	Amiard-Triquet *et al*. (1998b)
	0.55	1	0.55	Amiard *et al*. (2008)
	0.35	1	0.35	Amiard *et al*. (2008)
Digestive gland	2.25	1	2.25	Amiard *et al*. (1998a)
	8.04	0.2	1.61	Bebianno and Langston (1989)
	8	0.2	1.6	Bebianno and Langston (1991)
	8.8	0.2	1.76	Amiard-Triquet *et al*. (1998b)
	1.8	1	1.8	Pellerin and Amiard (2009)
	1.6	1	1.6	Geffard *et al*. (2005)
Gills	0.3	1	0.3	Amiard *et al*. (1998)
	2.2	0.2	0.44	Bebianno and Langston (1991)
	1.7	0.2	0.34	Amiard-Triquet *et al*. (1998b)
	8	0.08	0.63	Geret *et al*. (2002)
	0.23	1	0.23	Geffard *et al*. (2005)

Table 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 0.8 (water content; see e.g. Williams, 1970) and from protein-standardised values using a factor 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 ww)	Reference
Whole animal	12.1	0.2	2.4	Bebianno and Machado (1997)
	1.21	1	1.21	Raspor *et al*. (1999)
	3.21	0.2	0.64	Bebianno and Langston (1992)
	0.5	1	0.5	Mourgaud *et al*. (2011)
Digestive gland	4.09	1	4.09	Raspor *et al*. (1999)
	2.1	1	2.1	Pavicic *et al*. (1993)
	45	0.08	3.56	Zorita *et al*. (2007a)
Gills	0.62	1	0.62	Raspor *et al*. (1999)
	2.35	0.2	0.47	Bebianno *et al*. (1998)

Table 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 0.8 (water content; see e.g. Williams, 1970) and from protein-standardised values using a factor 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 ww)	Reference
M. edulis				
Whole animal	0.04	1	0.04	Brown *et al*. (2004)
Digestive gland	0.11	1	0.11	da Ros *et al*. (2007)
	0.16	1	0.16	Schiedek *et al*. (2006)
M. galloprovincialis				
Whole animal	20	0.08	1.6	Funes *et al*. (2006)
	0.45	1	0.45	Domouthsidou *et al*. (2004)
Digestive gland	0.3	1	0.3	Viarengo *et al*. (2000)
	0.45	1	0.45	Domouthsidou *et al*. (2004)
	0.15	1	0.15	Donnini *et al*. (2007)
Gills	40	1	40	Hamer *et al*. (2008)

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 (Geffard *et al*., 2005; Raspor *et al*., 2004; Zorita *et al*., 2007b). However, other studies have found higher values the autumn (Pellerin and Amiard, 2009). This may be due to 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.

Assessment criteria

The medians or averages from different studies with the three tissues were remarkably similar for M*. edulis*; provisional Background Assessment Criteria (BACs) were constructed using the 90 percentile of averages/medians from literature: Whole body: 0.6 µg/g ww; digestive gland 2.0 µg/g ww and gills 0.6 µg/g ww. These values comprise medians for a full seasonal cycle.

BACs for *M. galloprovincialis* generated in a similar way were: Whole body: 2.0 µg/g ww; digestive gland 3.9 µg/g ww and gills 0.6 µg/g ww. As above, the values are medians for a seasonal cycle.

MT concentrations measured using the sulphydryl method produced results very different to those found using differential pulse polarography; no assessment criteria have been established for this method.

References

Aceto, S. *et al*. 2011. The metallothionein genes of *Mytilus galloprovincialis*: Genomic organization, tissue expression and evolution. Marine Genomics, 4, 61–68.

Amiard, J.-C., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P.S. 2006. Metallothioneins in aquatic invertebrates: their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160–202.

Amiard, J.C., Geffard, A., Amiard-Triquet, C. 1998a. La metallothioneine chez la moule *Mytilus edulis* comme biomarqueur de pollution métallique: variabilité entre sites, saisons et organes. J. Rech. Oceanogr. 23, 25–30.

Amiard-Triquet, C., Rainglet, F., Larroux, C., Regoli, F., Hummel, H. 1998b. Metallothionein in Arctic bivalves. Ecotox. Environ. Saf. 41, 96–102.

Amiard, J.-C. *et al*. 2008. Influence of field and experimental exposure of mussels (*Mytilus* sp.) to nickel and vanadium on metallothionein concentration. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 147, 378–385.

Bebianno, M.J. *et al*. 1991. Metallothionein induction in *Mytilus edulis* exposed to cadmium. Mar.Biol., 108, 91–96.

Bebianno, M.J., Langston, W.J. 1989. Quantification of metallothionein in marine invertebrates using differential pulse polarography. Portugal Electrochim. Acta 7, 59–64.

Bebianno, M.J., Langston, W.J. 1992. Cadmium induction of metallothionein synthesis in *Mytilus galloprovincialis*. Comp. Biochem. Physiol. C 103, 79–85.

Bebianno, M.J., Langston, W.J. 1998. Cadmium and metallothionein turnover in different tissues of the gastropod Littorina littorea. Talanta 46, 301–313.

Bebianno, M.J., Machado, L.M. 1997. Concentrations of metals and metallothioneins in *Mytilus galloprovincialis* along the south coast of Portugal. Mar. Pollut. Bull. 34, 666–671.

Brown, R.J., Galloway, T.S., Lowe, D., Browne, M.A., Dissanayake, A., Jones, M.B., Depledge, M.H. 2004. Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. Aquat. Toxicol. 66, 267–278.

Castillo, E. del *et al*. 2008. Nuclear and cytosolic distribution of metallothionein in the blue mussel *Mytilus edulis* L. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 151, 46–51.

Da Ros, L. *et al*. 2007. Lysosomal responses and metallothionein induction in the blue mussel *Mytilus edulis* from the south-west coast of Iceland. Environment International, 33, 362–369.

Dare, P.J. *et al*. 1975. Seasonal changes in flesh weight and biochemical composition of mussels (*Mytilus edulis* L.) in the Conwy Estuary, North Wales. Journal of Experimental Marine Biology and Ecology, 18, 89–97.

Domouhtsidou, G.P. *et al*. 2004. Lysosomal membrane stability and metallothionein content in *Mytilus galloprovincialis* (L.), as biomarkers: Combination with trace metal concentrations. Marine Pollution Bulletin, 48, 572–586.

Dondero, F. *et al*. 2005. Quantitative PCR analysis of two molluscan metallothionein genes unveils differential expression and regulation. Gene, 345, 259–270.

Donnini, F. *et al*. 2007. A biological and geochemical integrated approach to assess the environmental quality of a coastal lagoon (Ravenna, Italy). Environment International, 33, 919–928.

Funes, V. *et al*. 2006. Ecotoxicological effects of metal pollution in two mollusc species from the Spanish South Atlantic littoral. Environmental Pollution, 139, 214–223.

Geffard, A. *et al*. 2005. Do seasonal changes affect metallothionein induction by metals in mussels, *Mytilus edulis*? Ecotoxicology and Environmental Safety, 61, 209–220.

Géret, F. *et al*. 2002. Influence of metal exposure on metallothionein synthesis and lipid peroxidation in two bivalve molluscs: the oyster (*Crassostrea gigas*) and the mussel (*Mytilus edulis*). Aquat. Living Resour., 15, 61–66.

Hamer, B. *et al*. 2008. Effect of hypoosmotic stress by low salinity acclimation of Mediterranean mussels *Mytilus galloprovincialis* on biological parameters used for pollution assessment. Aquatic Toxicology, 89, 137–151.

Ivankovic, D. *et al*. 2005. Evaluation of the *Mytilus galloprovincialis* Lam. digestive gland metallothionein as a biomarker in a long-term field study: Seasonal and spatial variability. Marine Pollution Bulletin, 50, 1303–1313.

Kijewski, T. *et al*. 2011. Distribution of *Mytilus* taxa in European coastal areas as inferred from molecular markers. Journal of Sea Research, 65, 224–234.

Leignel, V. *et al*. undefined. Isolation and characterization of *Mytilus edulis* metallothionein genes. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 142, 12–18.

Mackay, E.A. *et al*. 1990. Polymorphism of cadmium-induced mussel metallothionein. Experientia, 46, A36.

Mourgaud, Y. *et al*. 2011. Metallothionein concentration in the mussel *Mytilus galloprovincialis* as a biomarker of response to metal contamination: validation in the field. Biomarkers, 7, 479–490.

Olafson, R.W. *et al*. 1974. Isolation of Heavy Metal Binding Proteins from Marine Vertebrates. Mar.Biol., 28, 83–86.

Pavicic, J. *et al*. 1989. Determination of Cd-binding proteins similar to metallothioneins in the digestive gland of *Mytilus galloprovincialis* in relation to the preliminary treatment of the sample. Period.biol., 911, 213–224.

Pavicic, J., Raspor, B., Marticic, D. 1993. Quantitative determination of metallothionein-like proteins in mussels. Methodological approach and field evaluation. Mar. Biol. 115, 435–444.

Pellerin, J. *et al*. 2009. Comparison of bioaccumulation of metals and induction of metallothioneins in two marine bivalves (*Mytilus edulis* and *Mya arenaria*). Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 150, 186–195.

Raspor, B., Pavicic, J., Kozar, S., Kwokal, Z., Paic, M., Odzak, N., Ujevic, I., Kjakovic, Z. 1999. Assessment of metal exposure of marine edible mussels by means of a biomarker. In: Klaassen, C. (Ed.), Metallothionein IV. Birkhauser, Basel, pp. 629–632.

Raspor, B., Dragun, Z., Erk, M., Ivankovic, D., Pavicic, J. 2004. Is the digestive gland of *Mytilus galloprovincialis* a tissue of choice for estimating cadmium exposure by means of metallothioneins? Sci. Total Environ. 333, 99–108.

Roesijadi, G. *et al*. 1988. Immunochemical quantification of metallothioneins of a marine mollusc. Can.J.Fish.aquat.Sci., 45, 1257–1263.

Schiedek, D. *et al*. 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. Marine Pollution Bulletin, 53, 387–405.

Soazig, L. *et al*. 2003. Potential use of the levels of the mRNA of a specific metallothionein isoform (MT-20) in mussel (*Mytilus edulis*) as a biomarker of cadmium contamination. Marine Pollution Bulletin, 46, 1450–1455.

Vergani, L. *et al*. 2007. Molecular characterization and function analysis of MT-10 and MT-20 metallothionein isoforms from *Mytilus galloprovincialis*. Archives of Biochemistry and Biophysics, 465, 247–253.

Viarengo, A. *et al*. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Marine Environmental Research, 44, 69–84.

Viarengo, A. *et al*. 2000. Critical evaluation of an intercalibration exercise undertaken in the framework of the MED POL biomonitoring program. Marine Environmental Research, 49, 1–18.

Zorita, I. *et al*. 2007a. Tissue- and cell-specific expression of metallothionein genes in cadmium- and copper-exposed mussels analyzed by in situ hybridization and RT-PCR. Toxicology and Applied Pharmacology, 220,186–196.

Zorita, I. *et al*. 2007b. Assessment of biological effects of environmental pollution along the NW Mediterranean Sea using mussels as sentinel organisms. Environmental Pollution, 148, 236–250.

Chapter 11: Micronucleus assay as a tool for assessing cytogenetic/DNA damage in marine organisms

Background

Micronuclei (MN) consist of acentric fragments of chromosomes or whole chromosomes which 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 due to spindle apparatus malfunction, the lack or damage of 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 which can generate not only whole chromatids that are left out of the nuclei, thus forming MN, but also can form multinucleated cells in which each nucleus would contain a different number of chromosomes (Serrano-García and Montero-Montoya, 2001). Thus, the scoring during interphase provides a measure of genotoxicity both in the field and also specifically through genotoxic compound exposure in the laboratory due to clastogens and/or aneugens (Al-Sabti and Metcalfe, 1995; Heddle *et al.*, 1991). In addition, there are direct indications that MN additionally may be formed via a nuclear budding mechanism in the interphase of cell division. The formation of such type MN reflects in an unequal capacity of the organisms to expel damaged, amplified, failed replicated or improperly condensed DNA, chromosome fragments without telomeres and centromeres from the nucleus (Lindberg *et al.*, 2007).

The micronuclei assay involves the scoring of the cells which contain one or more micronuclei 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, de Raat (1982) were the first to successfully apply the assay to aquatic species when they demonstrated the induction of micronuclei in erythrocytes of the eastern mudminnow (*Umbra pygmaea*) following waterborne exposure to the known mutagen ethyl methanosulphate (EMS). Since these initial experiments, other studies have validated the detection of micronuclei as a suitable biomarker of genotoxicity in a wide range of both vertebrate and invertebrate species (for review see Chaudhary *et al.*, 2006; Udroiu *et al.*, 2006; Bolognesi and Hayashi, 2011). In fish most studies have utilised circulating erythrocytes (blood) cells but can also be sampled from a number of tissues, such as liver, kidney, gill or fin epithelium (Archipchuk and Garanko, 2005; Baršienė *et al.*, 2006a; Rybakovas *et al.*, 2009).

Environmental genotoxicity levels in organisms from 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.*, 2006b; Magni *et al.*, 2006; Fernandez *et al.*, 2011). Concerns about the environmental genotoxicity in an oil and gas industrial areas of the North Sea were raised when comparatively high levels of micronuclei incidences were detected in mussels *Mytilus edulis* and Atlantic cod *Gadus morhua* caged closely to the oil platforms (Hylland *et al.*, 2008). Increased environmental genotoxicity and cytotoxicity has 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 (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; Baršienė and Andreikėnaitė, 2007; Andreikėnaitė, 2010) and from Arctic zones (Baršienė *et al.*, unpublished data).

The frequency of the observed micronuclei may be considered as a suitable index of accumulated genetic damage during the cell lifespan providing a time integrated response of an organism's exposure to contaminant mixtures. Depending on the life-span of each cell type and on their mitotic rate in a particular tissue, the micronuclei frequency may provide early warning signs of cumulative stress (Bolognesi and Hayashi, 2011). The exposure of caged mussels in the Genoa harbour, heavily polluted by aromatic hydrocarbons showed a continuous increase of micronuclei in mussel gill cells reaching a plateau after a month of caging (Bolognesi *et al.*, 2004). After 30 days caging of mussels at the Cecina estuary in Tyrrhenian coast, 2-fold increase of MN incidences in gill cells has been observed (Nigro *et al.*, 2006). The 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 Haven oil ship sinking zone using the MN test in caged mussels ten years after the oil spill (Bolognesi *et al.*, 2006b). In this respect, increase in micronuclei frequency represents a time integrated response to cumulative stress.

Short description of methodology

Target species

Micronuclei 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 (as European flounder, dab, Atlantic cod or red mullet), which are routinely used in biomonitoring programmes and assess contamination along western European marine waters (see Table 1). However, the MN assay may 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 characterised by an elevated number of small chromosomes (Udroiu *et al.*, 2006). Thus, in certain cases micronuclei 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 cellular 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), the fish Arctic rockling *Omogadus argentus* and amphipods *Eurythenes gryllus* are suitable species. In equatorial regions of the Atlantic, indicator fish species *Brachydetrius aurectus*, *Synoglosus senegalensis*, *Cynoponticus ferox* are available for the MN analysis (Baršienė IRIS reports for Deepvann and Anquilla reports).

Target tissues

The majority of studies to date have used haemolymph and gill cells of molluscs and peripheral blood cells of fish for the MN analysis (Bolognesi and Hayashi, 2011). There are other studies (albeit limited) available describing the use of blood cells of fish in other tissues, such as liver, kidney and gills (Baršienė *et al.*, 2006a; Rybakovas *et al.*, 2009), and also other cells (fin cells) (Archipchuk 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 easy 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 cold blood erythrocytes and erythrocytes from different hemopoetic tissues (liver, kidney, gill and spleen) differ significantly after three weeks exposure to Stafjord B

crude oil. In multiple laboratory exposures (108 exposure groups of cod), developing liver and kidney erythrocytes were 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.*, 2005b, 2006a). Liver as a target organ can also be used in in situ exposures with turbot and halibut (caging or laboratory) (Baršienė, IRIS reports on BioSea, PROOF, WCM projects).

Sample and cell scoring size

The detected MN frequency in fish erythrocytes is approximately 6-10 times lower than in mussels and clams. The large inter-individual variability associated to the low baseline frequency for this biomarker confirming the need for the scoring of a consistent number of cells in an adequate number of animals for each study point. Sampling size in most of studies conducted with mollusc species have been scoring 1000-2000 cells per animal (Izquierdo *et al.*, 2003; Hagger *et al.*, 2005; Bolognesi *et al.*, 1996, 2004, 2006a; Magni *et al.*, 2006; Baršienė *et al.*, 2006a, 2006b, 2008b, 2010a, 2010b; Kopecka *et al.*, 2006; Nigro *et al.*, 2006; Schiedek *et al.*, 2006; Francioni *et al.*, 2007; Siu *et al.*, 2004; Koukouzika and Dimitriadis, 2005, 2008) and previous reviews have suggested that when using fish erythrocytes at least 2000-4000 cells should be scored per animal (Udroiu *et al.*, 2006; Bolognesi *et al.*, 2006). Previously scorings of 5000-10 000 fish erythrocytes where used for a 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.

Mussel sampling size in MN assays range from 5 to 20 mussels per site as reported in the literature (Venier and Zampieron, 2005; Bolognesi *et al.*, 2004; Baršienė *et al.*, 2004, 2006e, 2008a, 2008b; Francioni *et al.*, 2007; Siu *et al.*, 2004). Evidence suggests that a sample size of ten 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, due to 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; Baršienė *et al.*, unpublished results).

MN identification criteria

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

- The size of MN is smaller than 1/3 of the main nucleus.
- Micronuclei 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.
- Micronuclei are not connected to the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.

After sampling and cell smears preparation, slides should be coded. To minimize technical variation, the blind scoring of micronuclei should be performed without knowledge of the origin of the samples. Only cells with intact cellular and nuclear membrane 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 highest quality (good staining, non-overlapping cells). Scoring of micronuclei should then be undertaken at 1000x magnification.

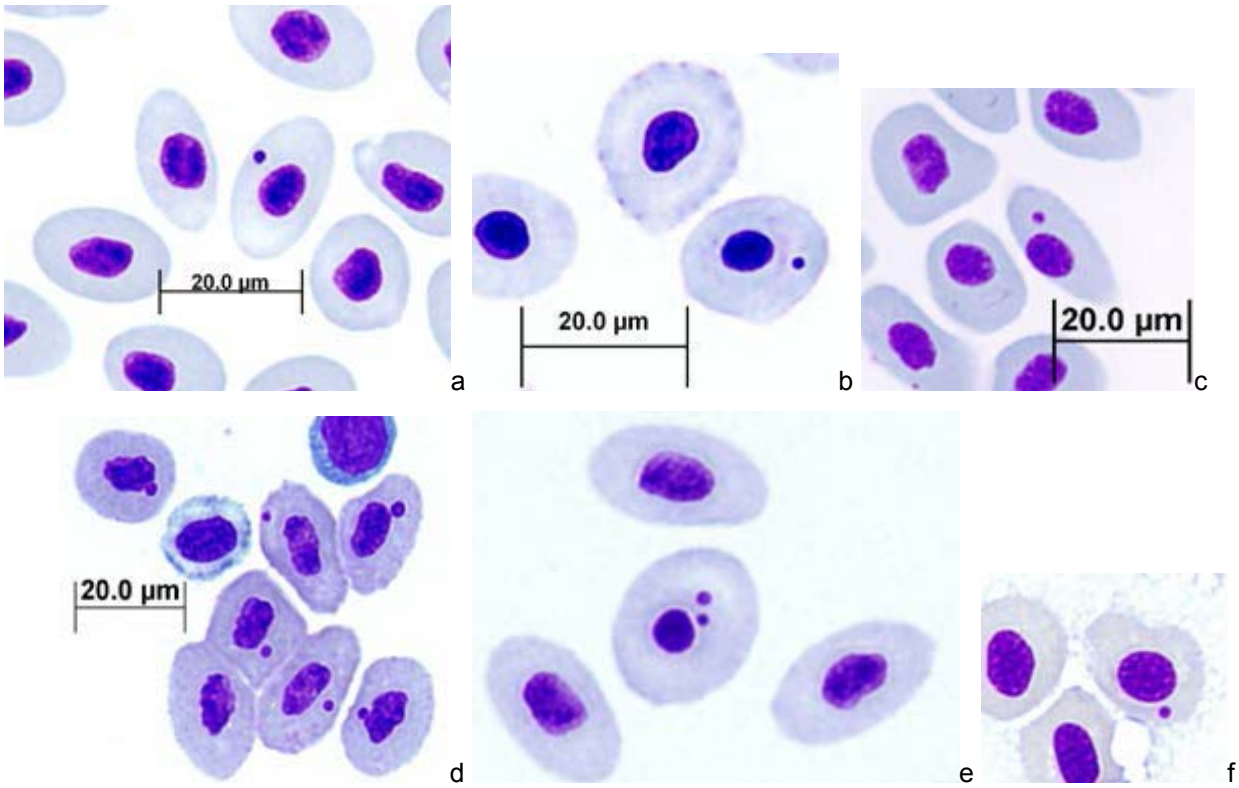

Image A. Micronuclei in blood erythrocytes of *Platichthys flesus* (a), *Limanda limanda* (b), *Zoarces viviparous* (c), *Clupea harrengus* (d), two MN in *Limanda limanda* (e) and MN liver erythrocytes of *Gadus morhua* (f). Images from NRC database.

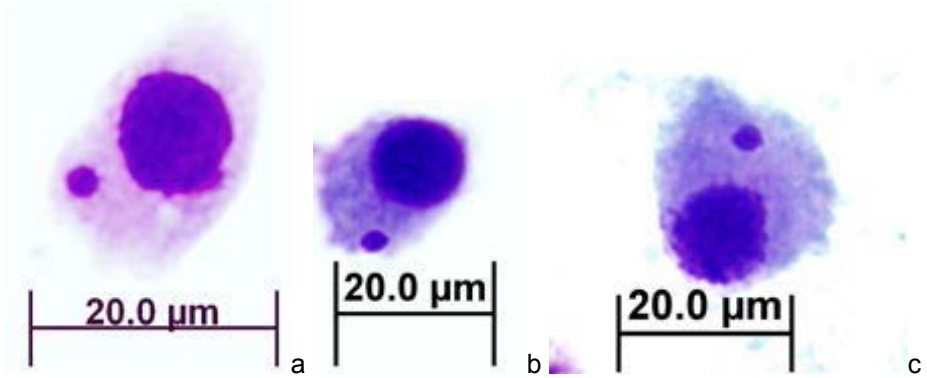

Image B. Micronuclei in gill cells of *Mytilus edulis* (a), *Macoma baltica* (b) and in haemocyte of *Chlamys islandica* (c). Images from NRC database.

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 description of genetic risk zones, or ecosystem health assessments.

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 showed also

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 due to high water temperatures in the autumn (Brunetti *et al.*, 1988). Additionally, it has been reported that increases in water temperature (4-37^{o}C) can increase the ability of genotoxic compounds to damage DNA (Buschini *et al.*, 2003).

Types of cells

MN may be seen in any type of cell, both somatic and germinal and thus the micronucleus test can be carried out in any active tissue. Nevertheless there are some limitations using different types of cells, for example, agranular and granular haemocytes in mussels. There are also differences between MN induction level 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 erythrocytes removal in the spleen (Udroiu *et al.*, 2006) like mammals do.

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 Sea. 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 Gulfs of Bothnia, Finland, Riga and Lithuanian EZ (Baršienė *et al.*, unpublished data).

Size

Since the linear regression analysis of animal's length and induction of MN shows that the size could be a confounding factor, sampling of organisms with similar sizes should take place (Baršienė *et al.*, unpublished data). It should also be noted that size is not always indicative of age and therefore age could also potentially affect the response of genotoxicity in the fish.

Diet

Results have shown that MN formation was not influenced in mussels who were maintained under simple laboratory conditions without feeding (Baršienė *et al.*, 2006e).

Ecological relevance

Markers of genotoxic effects reflect damage to genetic material of organisms and thus get a lot of attention (Moore *et al.*, 2004). Different methods have been developed for the detection of both double- and single-strand breaks of DNA, DNA-adducts, micronuclei 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). Micronucleus formation indicates chromosomal breaks, known to result in teratogenesis (effects on offspring) in mammals. There is however limited knowledge of relationships between micronucleus formation and effects on offspring in aquatic organisms. With a growing concern over the presence of genotoxins in the aquatic media, the application of cytogenetic assays on ecologically relevant species offers the chance to perform early tests on health in relation to exposure to contaminants.

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 micronuclei (MN) and other abnormal nuclear formations in different fish and bivalve species inhabiting various sites of the North Sea, Baltic Sea, Atlantic Ocean and Barents Sea. These studies revealed the relevance of environmental genotoxicity levels in ecosystem assessments. Nature Research Center established a large database on MN and other nuclear abnormalities in 13 fish species from the North Sea, Barents Seas and Atlantic Ocean, 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 1 and 2). 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 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 discharged 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 as well as in sites heavily polluted by copper or PAHs.

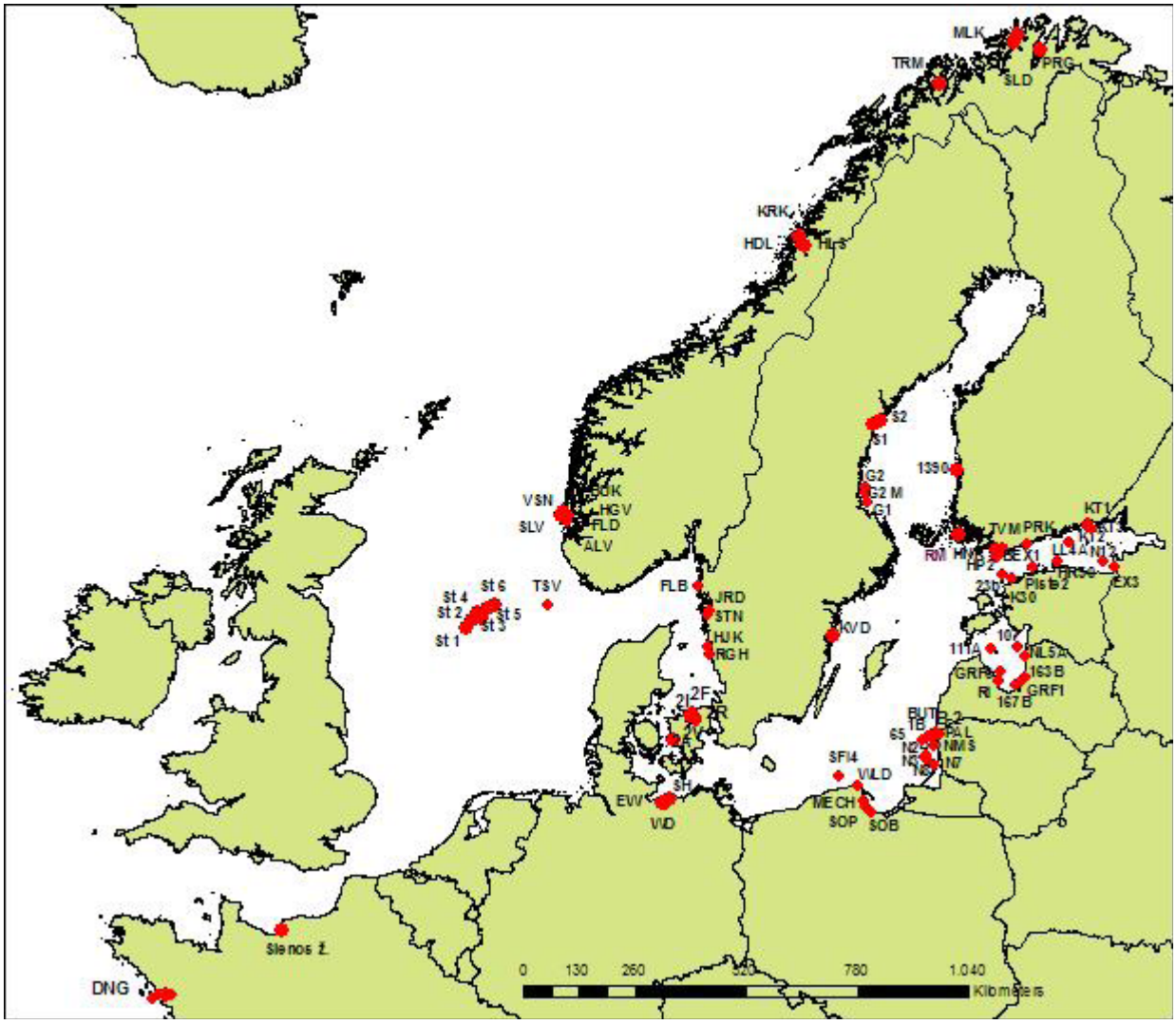

Figure 1. Sampling stations of bivalve molluscs for the micronuclei studies (NRC, Lithuania).

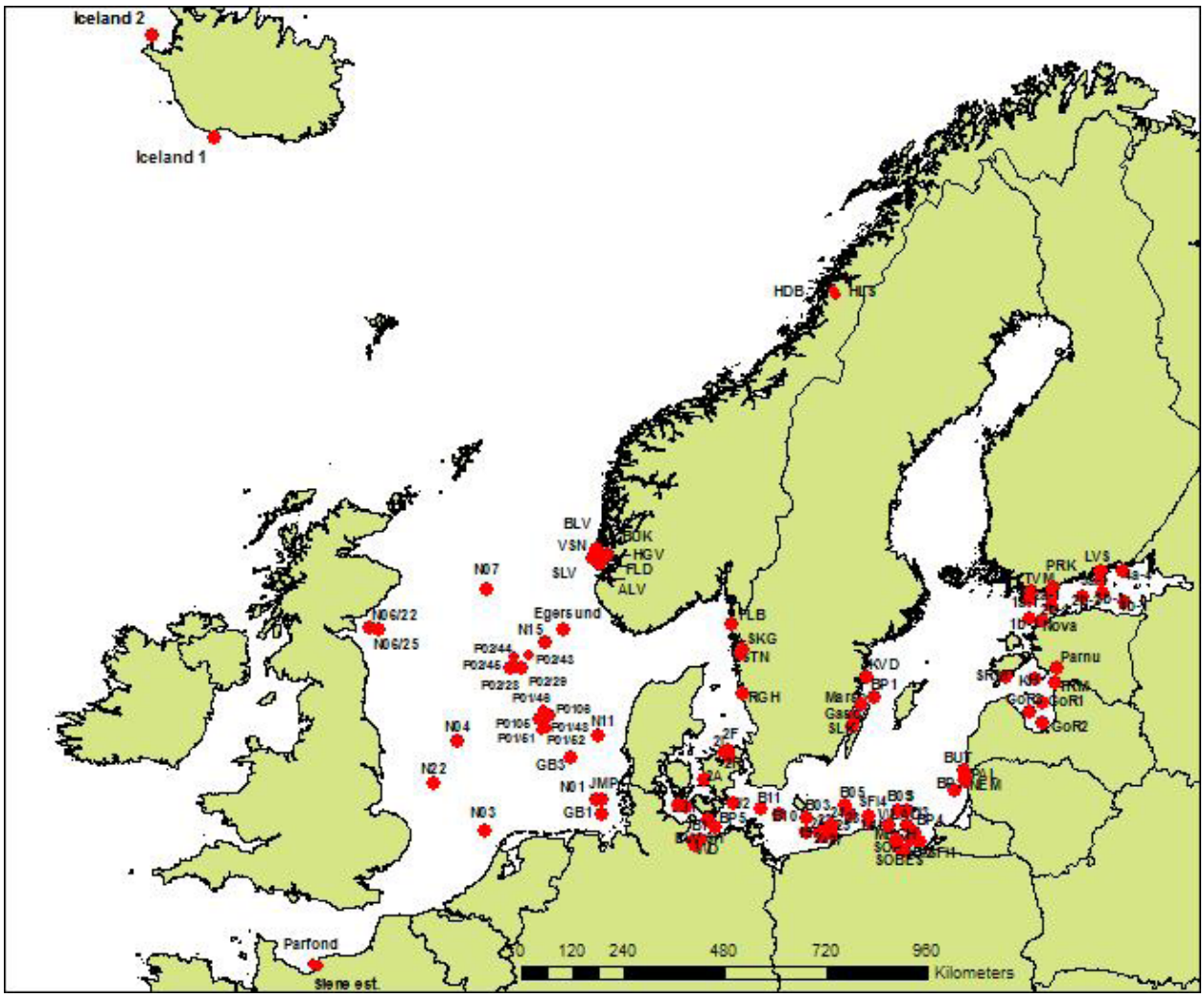

Figure 2. Sampling stations of fish species used for the micronuclei studies (NRC, Lithuania).

Background responses

Baseline or background frequency of MN can be defined as 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) has been suggested by Rybakovas *et al.* (2009) as a reference level in the peripheral blood erythrocytes of the flatfish flounder (*Platichthys flesus*) and dab (*Limanda limanda*) and also cod (*Gadus morhua*) - after analysing fishes 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 micronuclei in marine species sampled from field reference sites are summarized in Table 1. Additionally, the frequencies of MN in blood erythrocytes of fish and in gill cells of mussels deployed to the uncontaminated sites are shown (Table 2).

Table 1 The reference levels of micronuclei (MN/1000 cells) in European marine species in situ.

Species	Tissue	Location	Response MN/1000 cells	Reference
Mytilus galloprovincialis	Gills	Adriatic and Tyrrhenian Sea	1.0-at 15 °C 2.0-at 15-20 °C 3.0-at above 20 °C	Brunetti *et al.*, 1992
M. galloprovincialis	Haemolymph	Mediterranean coast	4.2 ± 0.7	Burgeot *et al.*, 1996
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	Fernandez *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	Baršienė *et al.*, 2004
M. edulis	Gills	North Sea (Goteborg 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.*, 2006b
M. trossulus	Gills	Baltic Sea	2.07 ± 0.32	Baršienė *et al.*, 2006b; Kopecka *et al.*, 2006
Macoma baltica	Gills	Baltic Sea	0.53-1.28	Baršienė *et al.*, 2008b, unpublished data
M baltica	Gills	Stockholm archipelago	0.4	Smolarz, 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	Baršienė *et al.*, unpublished data
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	Kohler, 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	Lyons, unpublished
Zoarces viviparus	Blood erythrocytes	Baltic Sea	0.02 ± 0.02	Baršienė *et al.*, unpublished data
Gadus morhua	Blood, kidney erythrocytes	North Sea	0.03 ± 0.02	Rybakovas *et al.*, 2009
G. morhua	Blood, kidney	Baltic Sea	0.03 ± 0.02	Rybakovas *et al.*, 2009

Species	Tissue	Location	Response MN/1000 cells	Reference
	erythrocytes			
Clupea harrengus	Blood erythrocytes	Baltic Sea	0.03 ± 0.03	Baršienė *et al.*, unpublished data
Symphodus melops	Blood erythrocytes	North Sea	0.08 ± 0.04	Baršienė *et al.*, 2004
Scophthalmus maximus	Blood erythrocytes	Baltic Sea	0.10 ± 0.04	Baršienė *et al.*, unpublished data
Perca fluviatilis	Blood erythrocytes	Baltic Sea	0.06 ± 0.02	Baršienė *et al.*, 2005a; Baršienė *et al.*, unpublished data
Mugil cephalus	Blood erythrocytes	Mediterranean coast, Turkey	0.82-2.07	Çavaş, Ergene-Gozukara, 2005
M. cephalus	Gill cells	Mediterranean coast, Turkey	1.84-2.91	Çavaş, Ergene-Gozukara, 2005
Mullus barbatus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.33 [a]	Bolognesi, 2006a
Dicentrarchus labrax	Blood erythrocytes	La Spiezia Gulf (Italy)	0.75 [a]	Bolognesi, 2006a
Pagellus mormyrus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.4 [a]	Bolognesi, 2006a
Sargus sargus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25 [a]	Bolognesi, 2006a
Seriola dumerili	Blood erythrocytes	La Spiezia Gulf (Italy)	0.38 [a]	Bolognesi, 2006a
Serranus cabrilla	Blood erythrocytes	La Spiezia Gulf (Italy)	0.0 [a]	Bolognesi, 2006a
Sparus auratus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.12 [a]	Bolognesi, 2006[a]
Sphyraena sphyraena	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25 [a]	Bolognesi, 2006a
Trachurus trachurus	Blood erythrocytes	La Spiezia Gulf (Italy)	0.25 [a]	Bolognesi, 2006a
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 micronuclei per 1000 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 2. The reference levels of micronuclei (MN/1000 cells) in European marine organisms after caging in uncontaminated/reference sites in situ.

Species	Tissue	Location/exposure time	Response MN/1000 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.*, 2006b
M. galloprovincialis	Gills	Cecina estuary / 4 weeks	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 weeks	1.87 ± 0.43	Baršienė *et al.*, 2006d

Species	Tissue	Location/exposure time	Response MN/1000 cells	Reference
M. edulis	Gills	Karmsund (Norway) / 4 weeks	1.40 ± 0.29	Baršienė *et al.*, unpublished data
M. edulis	Heamolymph	North Sea, oil platforms (Norway) / 6 weeks	2.13 ± 0.48	Hylland *et al.*, 2008
M. edulis	Heamolymph	Seiland site (Norway) / 5.5 months	2.60 ± 0.21	Baršienė *et al.*, unpublished data
M. edulis	Heamolymph	Ekofisk oil platform, North Sea / 6 week	1.24 ± 0.37 (2006) 3.34 ± 0.28 (2008) 2.78 ± 0.50 (2009)	Brooks *et al.*, 2011
M. edulis	Heamolymph	Oil refinery (France, 2004)	3.20 ± 0.36	Baršienė *et al.*, unpublished data
M. edulis	Heamolymph	Oil refinery (France, 2006)	2.34 ± 0.37	Baršienė *et al.*, unpublished data
M. edulis	Heamolymph	Oil refinery (Mongstad, 2007) /100 days	2.90 ± 0.40	Baršienė *et al.*, unpublished data
M. edulis	Heamolymph	Sea Empress clean reference area (90 days)	0.75 ± 0.46	Lyons *et al.*, 1998
M. edulis	Heamolymph	Sea Empress clean reference area (110 days)	0.81 ± 0.36	Lyons *et al.*, 1998
Crassostrea gigas		Haven oil spill area/30 days	1.49 ± 0.79 [a]	Bolognesi *et al.*, 2006b
Gadus morhua	Liver erythrocytes	North Sea, oil platforms (Norway) / 5 weeks	0.12 ± 0.05	Hylland *et al.*, 2008
G. morhua	Liver erythrocytes	North Sea, oil platforms (Norway) / 6 weeks	0.27 ± 0.13	Baršienė *et al.*, unpublished data
Boops boops		Haven oil spill area / 30 days	0.6 ± 0.7 [a]	Bolognesi *et al.*, 2006b
Mulus barbatus		Haven oil spill area / 30 days	0.7 ± 0.6 [a]	Bolognesi *et al.*, 2006b
Uranoscopus scaber		Haven oil spill area / 30 days	1.1 ± 0.5 [a]	Bolognesi *et al.*, 2006b

a . number of micronuclei per 1000 studied cells

Additionally, the range of variation of the frequency of MN in blood erythrocytes of fish and gill cells of *M. galloprovincialis* is displayed in Table 3.

Table 3. The range of MN frequency fish (blood, liver, kidney erythrocytes), in mussels, clams, scallops, amphipods (haemolymph, gill and mantle cells) from different sites of 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, 2006b, 2006c, 2008b, 2010a; Schiedek *et al.*, 2006
Mytilus trossulus	5	Gills	2.07-6.70	Baršienė *et al.*, 2006b, 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	Fernandez *et al.*, 2011
Macoma balthica	29	Gills	0.53-11.23	Baršienė *et al.*, 2008b; Baršienė *et al.*, unpublished data
Chlamys islandica	3	Haemolymph	3.50-5.83	Baršienė *et al.*, unpublished data

Species	Number of sites studied	Tissue	MN frequency range, ‰	Reference
Eurythenes gryllus	2	Haemolymph	0.35-0.52	Baršienė *et al.*, unpublished data
Limanda limanda	3	Blood	≈ 2-5 [b]	Bresler *et al.*, 1999
L. limanda	26	Blood, kidney	0.02-1.22	Rybakovas *et al.*, 2009; Baršienė *et al.*, unpublished data
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; Baršienė *et al.*, unpublished data
Zoarces viviparus	40	Blood	0.02-0.81	Baršienė *et al.*, 2005a, Baršienė *et al.*, unpublished data
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 harrengus	32	Blood	0.03-0.92	Baršienė *et al.*, unpublished data
Melanogrammus aeglefinus	3	Liver	0.06-0.75	Baršienė *et al.*, unpublished data
Scophthalmus maximus	4	Blood, liver, kidney	0.10-0.93	Baršienė *et al.*, unpublished data
Perca fluviatilis	14	Blood	0.06-1.15	Baršienė *et al.*, 2005a; Baršienė *et al.*, unpublished data
Brachydetrius aurestus	3	Liver	0.28-0.85	Baršienė *et al.*, unpublished data
Synoglossus senegalensis	2	Liver	0.33-0.45	Baršienė *et al.*, unpublished data
Cynoponticus ferox	2	Liver	0.13-0.96	Baršienė *et al.*, unpublished data
Rhinobatos irvinei	1	Liver	0.50	Baršienė *et al.*, unpublished data
Omogadus argentus	2	Liver	0.23-0.47	Baršienė *et al.*, unpublished data

a. Frequency of micronuclei in cells

b. Frequency of micronuclei in erythrocytes

Assessment criteria

Assessment Criteria (AC) have been established by using data available from studies of molluscs and fish in the North Sea, northern Atlantic (NRC database) and Mediterranean area (Table 4). The background/threshold level of micronuclei incidences is calculated as the empirical 90% percentile (P90). Until more data becomes available, values should be interpreted from existing national data sets. It should be noted that these values are provisional and require further validation when data becomes available from the ICES database.

The 90% 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 the P90 percentile, 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 being reference stations (i.e. no known local sources of contamination or those areas which were not considered unequivocally as reference sites but as those less influenced from human and industrial activity).

ACs in bivalves *Mytilus edulis*, *Mytilus trossulus*, *Macoma balthica* and *Chlamys islandica* (data from MN analysis in 4371 specimens), in fish *Limanda limanda*, *Zoarces viviparus*, *Platichthys flesus*, *Symphodus melops*, *Gadus morhua*, *Clupea harrengus* 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 using data from five or more reference locations (Table 1).

ACs for mussel *Mytilus galloprovincialis* and fish 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 (SE Spain). Because significant sexual differences were not observed in red mullet, data of both genders were considered.

Table 4 Assessment criteria of MN frequency levels in different bivalve mollusc and fish species. BR=Background response; ER = Elevated response; N = number of specimens analysed.

Species	Size (cm)	Temperature (^{o}C)	Regional Area	Tissue	BR	ER	N
Mytilus edulis	3-4	11-17	Atlantic-North Sea	Haemolymph, gills	<2.51	>2.51	1280
M. edulis	1.5-3	8-18	Baltic Sea	Gills	<2.50	>2.50	1810
M. edulis caged for 4.6 weeks	3-4	7-9	North Sea	Haemolymph	<4.1	> 4.1	44
M. edulis caged for 4.6 weeks	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	>0.52	544
Limanda limanda	18-25	8-17	Baltic Sea	Erythrocytes	<0.49	>0.49	117
Platichtys flesus	20-28	15-17	Atlantic-North Sea	Erythrocytes	<0.33	>0.33	62
Platichtys flesus	17-39	10-17	Baltic Sea coastal	Erythrocytes	<0.29	>0.29	828
Platichtys 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

Quality Assurance

The micronucleus test showed 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 inter-species differences need further investigation. In 2009 an inter-laboratory comparison exercise was organised within the framework of the MED POL programme using *M. galloprovincialis* species. The results are expected by mid-2011.

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 are expanded to include material from all the commonly used bioindicator species in 2011/2012.

Scientific potential

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

References

Al-Sabati, K., Metcalfe, C.D. Fish micronuclei for assessing genotoxicity in water. Mutation Research 1995; 323: 121-35.

Andreikėnaitė, L. 2010. Genotoxic and cytotoxic effects of contaminants discharged from the oil platforms in fish and mussels. Summary of doctoral dissertation, 38p., Vilnius.

Arkhipchuk, V.V., Garanko, N.N. 2005. Using the nucleolar biomarker and the micronucleus test on in vivo fish fin cells. Ecotoxicology and Environmental Safety. Vol. 62. P. 42-52.

Bagni, G., Baussant, T., Jonsson, G., Baršienė, J., Mascini, M. 2005. Electrochemical device for the rapid detection of genotoxic compounds in fish bile samples. Analytical Letters, 38: 2639-2652.

Baršienė, J., Rybakovas, A. 2006e. Cytogenetic and cytotoxic effects in gill cells of the blue mussel *Mytilus edulis* from the Baltic coast and after 1-3-day maintenance in laboratory. Acta Zoologica Lituanica, 16: 191-197.

Baršienė, J., Andreikėnaitė, L. 2007. Induction of micronuclei and other nuclear abnormalities in blue mussels exposed to crude oil from the North Sea. Ekologija, 53, No 3: 9-15.

Baršienė, J., Andreikėnaitė, L., Garnaga, G., Rybakovas, A. 2008b. Genotoxic and cytotoxic effects in bivalve molluscs *Macoma balthica* and *Mytilus edulis* from the Baltic Sea. Ekologija, 54: 44-50.

Baršienė, J., Bjornstad, A., Andreikėnaitė, L. 2010b. Induction of micronuclei and other nuclear abnormalities in blue mussels *Mytilus edulis* after 1-, 2-, 4- and 8-day treatment with crude oil from the North Sea. Ekologija, 56, 3-4: 116-123.

Baršienė, J., Bjornstad, A., Rybakovas, A., Šyvokienė, J., Andreikėnaitė, L. 2010a. Environmental genotoxicity and cytotoxicity studies in mussels and fish inhabiting northern Atlantic zones impacted by aluminium industry. Ekologija, 56, No 3-4: 124-131.

Baršienė, J., Dedonytė, V., Rybakovas, A., Andersen, O-K. 2005b. Induction of micronuclei in Atlantic cod (*Gadus morhua*) and turbot (*Scophthalmus maximus*) after treatment with bisphenol A, diallyl phthalate and tetrabromodiphenyl ether-47. Ekologija No 4, 1-7.

Baršienė, J., Dedonytė, V., Rybakovas, A., Andreikėnaitė, L., Andersen, O.K. 2006a. Investigation of micronuclei and other nuclear abnormalities in peripheral blood and kidney of marine fish treated with crude oil. Aquatic Toxicology, 78, Supplement 1: S99-104.

Baršienė, J., Dedonytė, V., Rybakovas, A., Broeg, K., Forlin, L. , Gercken, J., Kopecka, J., Balk, L. 2005a. Environmental mutagenesis in different zones of the Baltic Sea. Acta Zoologica Lituanica, 15: 90-95.

Baršienė, J., Lazutka, J., Šyvokienė, J., Dedonytė, V., Rybakovas, A., Bjornstad, A., Andersen, O.K. 2004. Analysis of micronuclei in blue mussels and fish from the Baltic and the North Seas. Environmental Toxicology, 19: 365-371.

Baršienė, J., Lehtonen, K., Koehler, A., Broeg, K., Vourinen, P.J., Lang, T., Pempkowiak, J., Šyvokienė, J., Dedonytė, V., Rybakovas, A., Repečka, R., Vountisjarvi, H., Kopecka, J. 2006c. Biomarker responses in flounder (*Platichthys flesus*) and mussel (*Mytilus edulis*) in the Klaipėda-Būtingė area (Baltic Sea). Marine Pollution Bulletin, 53: 422-436.

Baršienė, J., Rybakovas, A., Andreikėnaitė, L. 2006d. Cytogenetic damage in marine organisms as a biomarker of environmental pollution. Metals in the Environment, 3rd Intern. Conf. in Lithuania. Vilnius 2006a, 156-157.

Baršienė, J., Rybakovas, A., Forlin, L., Šyvokienė, J. 2008a. Environmental genotoxicity studies in mussels and fish from the Goteborg area of the North Sea. Acta Zool. Lituanica, 18: 240-247.

Baršienė, J., Schiedek, D., Rybakovas, A., Šyvokienė, J., Kopecka, J., Forlin, L. 2006b. Cytogenetic and cytotoxic effects in gill cells of the blue mussel *Mytilus spp*. from different zones of the Baltic Sea. Marine Pollution Bulletin, 53: 469-478.

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., Pellegrini, D., Regoli, F. 2008. Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. Aquatic Toxicology, 89: 257-266.

Boller, K., Schmid, W. 1970. Chemische Mutagenese beim Sauger. Das Knochermark des Chinesischen Hamsters als *in vivo*-Test system. Haematologische Befunde nach Behandlung mit Trenimon. Humangenetik, 11: 35-54.

Bolognesi, C., Perrone, E., Roggieri, P., Pampanin, D.M., Sciutto, A. 2006a. Assessment of micronuclei induction in peripheral erythrocytes of fish exposed to xenobiotics under controlled conditions. Aquatic Toxicology, 78, Supplement 1: S93-S98.

Bolognesi, C., Perrone, E., Roggieri, P., Sciutto, A. 2006b. Bioindicators in monitoring long term genotoxic impact of oil spill: Haven case study. Marine Environ. Res., 62: S287-S291. Bolognesi C., Hayashi M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26: 205-213.

Bolognesi, C. and Hayashi, M. 2011. Micronucleus assay in aquatic animals. Mutagenesis, 26: 205-213.

Bolognesi, C., Frenzilli, G., Lasagna, C., Perrone, E., Roggieri, P. 2004. Genotoxicity biomarkers in *Mytilus galloprovincialis*: wild versus caged mussels. Mutation Research. 552: 153-162.

Bolognesi, C., Rabboni, R. and Roggieri, P. 1996. Genotoxicity biomarkers in *M. Galloprovincialis* as indicators of marine pollutants. Comparative Biochemistry and Physiology 113C (2): 319-323.

Bresler, V., Bissinger, V., Abelson, A., Dizer, H., Sturm, A., Kratke, R., Fischelson, L., Hansen, P.-D. 1999. Marine molluscs and fish as biomarkers of pollution stress in littoral regions of the Red Sea, Mediterranean Sea and North Sea. Helgoland Marine Research, 53: 219-243.

Brooks, S.J., Harman, C., Grung, M., Farmen, E., Ruus, A., Vingen, S., Godal, B.F., Baršienė, J., Andreikėnaitė, L., Skarphedinsdottir H., Liewenborg B., Sundt R.C. 2011. Water column monitoring of the biological effects of produced water from the Ekofisk offshore oil installation from 2006 to 2009. Journal of Toxicology and Environmental Health, Part A, 74: 582-604.

Brunetti, R., Gabriele, M., Valerio, P. and Fumagalli, O. 1992. The micronucleus test: temporal pattern of the base-line frequency in *Mytilus galloprovincialis* Lmk. Marine Ecol. Prog. 83: 75-78.

Brunetti, R., Majone, F., Gola, I. and Beltrame, C. 1988. The micronucleus test: examples of application to marine ecology. Marine Ecol. Prog., 44: 65-68.

Buschini, A, Carboni, P, Martino, A, Poli, P, Rossi, C. 2003. Effects of temperature on baseline and genotoxicant-induced DNA damage in haemocytes of *Dreissena polymorpha*. Mutation Research, 537: 81-92.

Burgeot, T., Woll, S. and Galgani, F. 1996. Evaluation of the micronucleus test on *Mytilus galloprovincialis* for monitoring applications along French coasts. Marine Pollution Bulletin, Volume 32, No. 1: 39-46.

Carrasco, K.R., Tilbury K.L., Myers, M.S. 1990. Assessment of the piscine micronucleus test as an *in situ* biological indicator of chemical contaminant effects. Canadian Journal Fishery and Aquatic Sciences, 47: 2123-2136.

Çavaş, T., Ergene-Gözükara, S. 2005. Micronucleus test in fish cells: a bioassay for *in situ* monitoring of genotoxic pollution in the marine environment. Environmental and Molecular Mutagenesis, 46: 64-70.

Chaudhary, R., Pandy, S., Kushwaha, B., Gaur, K.K., Nagpure, N.S. 2006. Fish micronucleus assay: A sensitive tool for ecogenotoxicity studies. Journal of Ecophysiology and Occupational Health, 6 (3-4): 143-147.

Dailianis, S., Domouhtsidou, G.P., Raftopoulou, E., Kalayianni, M., Dimitriadis, V.K. 2003. Evaluation of neutral red retention assay, micronucleus test, acetylcholinesterase activity and a signal transduction molecule (cAMP) in tissues of *Mytilus galloprovincialis* (L.), in pollution monitoring. Marine Environmental Research, 56: 443-470.

Dixon, D.R., Dixon, L.R.J., Shillito, B., Gwynn, J.P. 2002. Background and induced levels of DNA damage in Pacific deep-sea vent palychaetes: the case for avoidance. Cah. Biol. Marine 43: 333-336.

Dolcetti, L. and Venier, P. 2002. Susceptibility to genetic damage and cell types in Mediterranean mussels. Marine Environmental Research, 54: 487-491.

Ergene, S., Çavaş, T., Celik, A., Koleli, N., Kaya, F., Karahan, A. 2007. Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): genotoxic damage in relation to water pollution. Ecotoxicology, 16: 385-391.

Fang, J.K.H., Wu, R.S.S., Yip, C.K.M., Shin, P.K.S. 2009. Power analysis for biomarkers in mussels for use in coastal pollution monitoring. Marine Pollution Bulletin, 58: 1152-1158.

Fenech, M. 2000. The in vitro micronucleus technique. Mutation Research 455: 81-95.

Fenech, M. 1993. The cytokinesis-block micronucleus technique: A detailed description of the method and Its application to genotoxicity studies in human populations. Mutation Research, 285, 35-44.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S., Zeiger, E. 2003. HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. Mutation Research, 534: 65-75.

Fernández, B., Campillo, J.A., Martínez-Gómez, C., Benedicto, J. 2011. Micronuclei and other nuclear abnormalities in mussels (*Mytilos galloprovincialis*) as biomarkers of cyto-genotoxic pollution in Mediterranean waters. Environmental and Molecular Mutagenesis. Accepted 25 January 2011. DOI 10-1002/em.20646.

Francioni, E., Wagener, A. de L.R., Scofield, A. de L., Depledge, M.H., Cavalier, B., Sette, C.B., Carvalhosa, L., Lozinsky, C., Mariath, R. 2007. Polycyclic aromatic hydrocarbon in inter-tidal mussel *Perna perna*: Space-time observations, source investigation and genotoxicity. Science of the Total Environment, Vol. 372 Issue 2/3: 515-531.

Gorbi, S., Lamberti, C.V., Notti, A., Benedetti, M., Fattorini, D., Moltedo, G., Regoli, F. 2008. An Ecotoxicological protocol with caged mussels, *Mytilus galloprovincialis*, for monitoring the impact of an offshore platform in the Adriatic Sea. Marine Environmental Research, 65: 34-49.

Hagger, J.A., Depledge, M.H., Galloway, T.S. 2005. Toxicity of tributyltin in the marine mollusc *Mytilus edulis*. Marine Pollution Bulletin, 51: 811-816.

Heddle, J.A. 1973. A rapid *in vivo* test for chromosomal damage. Mutation Research, 18: 187-190.

Heddle, J.A., Cimino, M.C., Hayashi, M., Romagna, F., Shelby, M.D., Tucker, J.D., Vanparys, P.H. and MacGregor, J.T. 1991. Micronuclei as an index of cytogenetic damage: past, present, and future. Environmental and Molecular Mutagenesis, 18: 277-291.

Hooftman, R.N., Raat, W.K. 1982. Induction of nuclear anomalies (micronuclei) in the peripheral blood erythrocytes of an eastern mudminnow *Umbra pygmaea* by ethyl methanesulphonate. Mutation Research, 104: 147-152.

Hylland, K., Tollefsen, K.-E., Ruus, A., Jonsson, G., Sundt, R.C., Sanni, S., Utvik, T.I.R., Johnsen, I., Pinturier, L., Balk, L., Baršienė, J., Marigomez, I., Feist, S.W., Borseth, J.F. 2008. Water column monitoring near oil installations in the North Sea 2001-2004. Marine Pollution Bulletin, 56: 414-429.

Izquierdo, J.I., Machado, G., Ayllon, F., d'Amico, V.L., Bala, L.O., Vallarino, E., Elias, R., Garcia-Vazquez, E. 2003. Assessing pollution in coastal ecosystems: a preliminary survey using the micronucleus test in the *Mytilus edulis*. Ecotoxicology and Environmental Safety 55: 24-29.

Kalpaxis, D.L., Teso, C., Xaplanteri, M.A., Dinos, G.P., Catsiki, A.V., Leotsinidis, M. 2004. Biomonitoring of Gulf of Patras, N. Peloponnesus, Greece. Application of a biomarker suite including evaluation of translation efficiency in *Mytilus galloprovincialis* cells. Environmental Research, 94: 211-220.

Klobučar, G.I.V., Štambuk, A., Hylland, K., Pavlica, M. 2008. Detection of DNA damage in haemocytes of *Mytilus galloprovincialis* in the coastal ecosystems of Kaštela and Trogir bays, Croatia. Sciences of the Total Environment, 405: 330-337.

Kopecka, J., Lehtonen, K., Baršienė, J., Broeg, K., Vuorinen, P.J., Gercken, J., Pempkowiak, J. 2006. Measurements of biomarker levels in flounder (*Platichthys flesus*) and mussel (*Mytilus trossulus*) from the Gulf of Gdańsk (southern Baltic). Marine Pollution Bulletin, 53: 406-421.

Koukouzika, N., Dimitriadis, V.K. 2005. Multiple biomarker comparison in *Mytilus galloprovincialis* from the Greece coast: "Lysosomal membrane stability, neutral red retention, micronucleus frequency and stress on stress". Ecotoxicology, 14:449-463.

Koukouzika, N., Dimitriadis, V.K. 2008. Aspects of the usefulness of five marine pollution biomarkers, with emphasis on MN and lipid content. Marine Pollution Bulletin, 56:941-949.

Lindberg, H.K., Wang, X., Järventaus, H., Falck, G.C.-M., Norppa, H., Fenech, M. 2007. Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes. Mutation Research, 617: 33-45.

Lyons, B.P. 1998. Methods for detecting genotoxic exposure in the aquatic environment. PhD thesis. Centre for Molecular Genetics and Toxicology, University of Wales Swansea.

Magni, P., De Falco, G., Falugi, C., Franzoni, M., Monteverde, M., Perrone, E., Sigro, M., Bolognesi, C. 2006. Genotoxicity biomarkers and acetylcholinesterase activity in natural populations of *Mytilus galloprovincialis* along a pollution gradient in the Gulf of Oristano (Sardinia, western Mediterranean). Environmental Pollution, 142: 65-72.

Martínez-Gómez, C., Navarrro, B., Fernández, J., Valdés and Benedicto, J. 2010. Evidence of genotoxic effects in red mullet populations from the Mediterranean coast of Spain. ICES Annual Science Conference. Nantes (France), 18-25 September 2010. Proceeding book, ICES CM 2010:/F31.

Moore, M.N., Depledge, M.H., Readman, J.W., Leonard, P. 2004. An integrated biomarker-based strategy for ecotoxicological evaluation of risk in environmental management. Mutation Research, 552:247-268.

Napierska, D., Baršienė, J., Mulkiewicz, E., Podolska, M., Rybakovas, A. 2009. Biomarker responses in flounder *Platichthys flesus* from the Polish coastal area of the Baltic Sea and application in biomonitoring. Ecotoxicology, 18: 846-859.

Nigro, M., Falleni, A., Del Barga, I., Scarcelli, V., Lucchesi, P., Regoli, F., Frenzilli, G. 2006. Cellular biomarkers for monitoring estuarine environments: Transplanted versus native mussels. Aquatic Toxicology, 77: 339-347.

Pampanin, D.M., Marangon, I., Volpato, E., Campesan, G., Nasci, C. 2005. Stress biomarkers and alkali-labile phosphates level in mussels (*Mytilus galloprovincialis*) collected in the urban area of Venice (Venice Lagoon, Italy). Environmental Pollution, 136: 103-107.

Pavlica, M., Podrug, M., Štambuk, A., Cvjetko, P., Klobučar, G.I.V. 2008. Seasonal Variability in Micronuclei Induction in Haemocytes of Mussels along the Eastern Adriatic Coast. Polish Journal of Environmental Studies, 17, No. 5: 765-771.

Pytharopoulou, S., Sazaklib, E., Grintzalisc, K., Georgiouc, C.D., Leotsinidis, M., Kalpaxis, D.L. 2008. Translational responses of *Mytilus galloprovincialis* to environmental pollution: Integrating the responses to oxidative stress and other biomarker responses into a general stress index. Aquatic Toxicology, 89: 18-27.

Rybakovas, A., Baršienė, J., Lang, T. 2009. Environmental genotoxicity and cytotoxicity in the offshore zones of the Baltic and North Seas. Marine Environmental Research, 68: 246-256.

Santos, M.M., Sole, M., Lima, D., Hambach, B., Ferreira, A.M., Reis-Henriques, M.A. 2010. Validating a multi-biomarker approach with the shanny *Lipophrys pholis* to monitor oil spills in European marine ecosystems. Chemosphere, 81, 6, 685-691.

Scarpato, R., Migliore, L., Barale, R. 1990. The micronucleus assay in *Anodonta cygnea* for the detection of drinking water mutagenecity. Mutation Research, 245: 231-237.

Schiedek, D., Broeg, K., Baršienė, J., Lehtonen, K.K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P.J., Dedonytė, V., Koehler, A., Balk, L., Schneider, R. 2006. Biomarker responses as indication of contaminant effects in blue mussel (*Mytilus edulis*) and female eelpout (*Zoarces viviparus*) from the southwestern Baltic Sea. Marine Pollution Bulletin, 53: 387-405.

Schmid, W. 1975. The micronucleus test. Mutation Research, 31:9-15.

Serrano-García, L., Montero-Montoya, R., 2001. Micronuclei and chromatid buds are the result of related genotoxic events. Environmental and Molecular Mutagenesis 38:38-45.

Siu, W.H.L., Cao, J., Jack, R.W., Wu R.S.S., Richardson, B.J., Xu, L. and Lam, P.K.S. 2004. Application of the comet and micronucleus assays to the detection of B a P genotoxicity in haemocytes of the greenlipped mussel (*Perna viridis*). Aquatic Toxicology, 66: 381-392.

Smolarz, K., Berger, A. 2009. Long-term toxicity of hexabromocyclododecane (HBCDD) to the benthic clam *Macoma balthica* (L.) from the Baltic Sea. Aquatic Toxicology, 95: 239-247.

Strunjak-Perovic, I., Topic, Popovic, N., Coz-Rakovac, R., Jadan, M. 2009. Nuclear abnormalities of marine fish erythrocytes. Journal of Fish Biology, 74 (10): 2239-2249.

Taleb, Z.M., Benali, I., Gherras, H., Ykhlef-Allal, A., Bachir-Bouiadjra, B., Amiard, J.C., Boutiba, Z. 2009. Biomonitoring of environmental pollution on the Algerian west coast using caged mussels *Mytilus galloprovincialis*. Oceanologia, 51 (1): 63-84.

Udroiu, I. 2006. The micronucleus test in piscine erythrocytes. Aquatic Toxicology, 79: 201-204.

Santos, M.M., Sole, M., Lima, D., Hambach, B., Ferreira, A.M., Reis-Henriques, M.A. 2010. Validating a multi-biomarker approach with the shanny *Lipophrys pholis* to monitor oil spills in European marine ecosystems. Chemosphere, 81: 685-691.

Venier, P. and Zampieron, C. 2005. Evidence of genetic damage in grass gobies and mussels from the Venice lagoon. Environment International, 31: 1053-1064.

Wrisberg, M.N., Bilbo, C.M., Spliid, H. 1992. Induction of micronuclei in hemocytes of *Mytilus edulis* and statistical analysis. Ecotoxicology and Environmental Safety, 23: 191-205.

Chapter 12: Polycyclic Aromatic Hydrocarbon (PAH) metabolites in bile

Background

Analyses of PAH metabolites in fish bile have been used as a biomarker of exposure to PAH contamination since the early 1980s. The presence of metabolites in bile (and in urine) is the final stage of the biotransformation process whereby lipophilic compounds are transformed to 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 pre-treatment 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 samples while HPLC/F or GC-MS SIM is utilised for qualitative and quantitative measures (Ariese *et al*., 2005; Jonsson *et al*., 2003; Lin *et al*., 1996; Aas *et al*., 2000a, 2000b).

Bile is generally stored in the gall bladder prior to episodic release into the oesophagus where bile salts have a function to perform 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, which must be accounted for. The feeding status of fish has been shown 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 PAH 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 for estimating the actual biotic stress due to PAH exposure than the body burden data of the unmetabolised parent PAH compounds in tissues (most often liver). Despite this, body burden measurements are still more commonly used within monitoring studies than metabolite determination.

Dose-response (species specific)

The PAH compounds are metabolised rapidly in the organisms and it is the endpoint of this metabolisms that is measured in the bile. The compounds are measured 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. Since the 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 feed just before sampling, the gall bladder may become 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 digestion is important for the dose-response relationship. Fish generally have a very efficient metabolic excretion of most PAHs and it has been shown that most of the PAH will be excreted after 2 - 8 days following exposure. This means that the PAH metabolites determined in bile will represent exposures on the scale of days and, at most, two weeks.

It has been shown in several fields 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 make a dose-response relationship that can be used to quantify the 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) (Hylland, unpublished). This normalisation is not standardised because it has been shown to only explain parts of the variability, but it is recommended to be part of the explaining factors in the interpretation of results. In laboratory studies it is normal to stop the feeding some days before sampling to ensure the bile quality. In field sampling this can be taken into account by letting the fish go some days in tanks before sampling, but this has some logistical challenges.

Species sensitivity

The background level differs between species so it is important to establish good baseline before using new species. It may be expected that species with fatty livers*, i.e.* most gadoids, may metabolise 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 Background Assessment Criteria (BACs) and Environmental Assessment Criteria (EACs), though in some cases the resulting ACs are so similar that a combined AC for several species is justified (Table 3 below gives an example.)

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 cod sampled through 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 due to 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 PAH to the cellular compartments of liver cells. There are indications for seasonal differences between summer and winter values of PAH metabolites in dab (Kammann, 2007).

Background responses

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

Assessment criteria

Assessment Criteria for PAH metabolites such as BAC have been derived from reference sites (Table 3). EAC can be derived from toxicological experiment data by linking oil exposure and PAH metabolites in fish with DNA-adducts and fitness data (Skadsheim, A. 2004; Skadsheim *et al*., 2009), where the latter serves as the effect quantity for the calculation of the EAC presented in Table 3. 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.

Quality assurance

A general protocol outlining analytical strategies and their strengths as well as weaknesses has recently become available (Ariese *et al*., 2005). There have been international intercalibration exercises for the determination of PAH-metabolites in fish bile, arranged in collaboration between an EU-project and QUASIMEME[2]. Reference bile samples were generated as part of the aforementioned EU project and are now available through IRMM, JRC, Geel, Belgium (http://www.irmm.jrc.be/html/homepage.html). An intercalibration for PAH metabolites has been taking place in the framework of the EU funded BONUS project BEAST in 2010.

[2] QUASIMEME – organisation that offers quality assurance for chemical endpoints; http://www.quasimeme.org

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References

Aas, E., T. Baussant, *et al*. 2000a. PAH metabolites in bile, cytochrome P4501A and DNA adducts as environmental risk parameters for chronic oil exposure: a laboratory experiment with Atlantic cod. Aquatic Toxicology, 51: 241 258.

Aas, E., Beyer, J., Goksøyr, A. 2000b. Fixed wavelength fluorescence (FF) of bile as a monitoring tool for polyaromatic hydrocarbon exposure in fish: An evaluation of compound specificity, inner filter effect and signal interpretation. Biomarkers, 5: 9 23.

Ariese, F., Beyer, J., Jonsson, G., Visa, C.P., Krahn, M.M. 2005. Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile. ICES Techniques in Marine Environmental Sciences, 39: 1-41.

Beyer J, Sandvik M, Skåre JU, Egaas E, Hylland K, Waagbø R, Goksøyr A. 1997. Time- and dose-dependent biomarker responses in flounder (*Platichthys flesus* L.) exposed to benzo[a]pyrene, 2,3,3',4,4',5-hexachlorobiphenyl (PCB-156) and cadmium. Biomarkers, 2: 35 44.

Collier, T.K. and Varanasi, U. 1991. Hepatic activities of xenobiotic metabolizing enzymes and biliary levels of xenobiotics in English sole (*Parophrys vetulus*) exposed to environmental contaminants. Archives of Environmental Contamination and Toxicology, 20: 463 473.

Hylland, K., Ruus, A., Børseth, J.F., Bechmann, R., Baršienė, J., Grung, M., Tollefsen, K., Myhre, L.P. 2006. Biomarkers in monitoring - a review. NIVA-report 5205. 106 p.

Jonsson, G., Beyer, J., Wells, D., Ariese, F. 2003. The application of HPLC-F and GC-MS to the analysis of selected hydroxy polycyclic hydrocarbons in two certified fish bile reference materials. Journal of Environmental Monitoring, 5: 513 520.

Kammann U. 2007. PAH metabolites in bile fluids of dab (*Limanda limanda*) and flounder (*Platichthys flesus*) spatial distribution and seasonal changes. Environ Sci Pollut Res, 14(2): 102 108.

Lin, E.L.C., Cormier, S.M.Torsella, J.A., 1996. Fish biliary polycyclic aromatic hydrocarbon metabolites estimated by fixed-wavelength fluorescence: Comparison with HPLC-fluorescent detection. Ecotoxicology and environmental safety, 35: 16 23.

Ruus, A, Hylland, K, Green, N. 2003. Joint Assessment and Monitoring Programme (JAMP). Biological Effects Methods, Norwegian Monitoring 1997-2001. SFT Report no. 869/03. TA- 1948/2003, 139 p.

Skadsheim, A. 2004. Cod stocks exposed to crude oils: uptake, metabolism and biomarker effects. Technical report AM-2004/005, Akvamiljo, Randaberg, Norway.

Skadsheim, A., Sanni, S., Pinturier, L., Moltu, U.-E., Buffagni, M. and Bracco, L. 2009. Assessing and monitoring long-range-transported hydrocarbons as potential stressors to fish stocks. Deep-Sea Research Part II, 56(21 22): 2037 2043.

Vuorinen, P.J., Keinänen, M., Vuontisjärvi, H., Baršienė, J, Broeg, K., Förlin, L., Gercken, J., Kopecka, J., Köhler, A., Parkkonen, K., Pempkowiak, J., Schiedek, D. 2006. Use of biliary PAH metabolites as a biomarker of pollution in fish from the Baltic Sea Marine Pollution Bulletin, 53(8-9): 479-487.

Table 1 Overview of field and laboratory studies PAH metabolites measured by fixed fluorescence.

Species	Substance (lab/field)	Test concentrations/area	Exposure time	Metabolite	Baseline	Control or reference	exposed/control	reference/comments
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline					Aas *et al*., 2003
Cod (*Gadus morhua*)	Feral fish	Egersund	Baseline non polluted area	Naph type Pyren type BaP type	5.3 ug/ml 0.8 ug/ml 0.4 ug/ml			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Sleipner	Baseline polluted area?	Naph type Pyren type BaP type	6.1 ug/ml 1.0 ug/ml 0.5 ug/ml			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Statfjord	Baseline polluted area?	Naph type Pyren type BaP type	5.9 ug/ml 0.9 ug/ml 0.3 ug/ml			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Frøy, ceased installation 10 000 m (ref) 2000 m - 200 m	Baseline polluted area?	Naph type Pyren type BaP type		3.9 ug/ml 0.6 ug/ml 0.3 ug/ml	1.1 - 1.1 1.1 - 0.9 0.9 - 0.9	Beyer *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph type Pyren type BaP type	2.15 ug/g 1.63 ug/g 0.69 ug/g			Sundt, 2002
Cod (*Gadus morhua*)	Feral fish	Barents Sea	Baseline	Naph type Pyren type BaP type	5.8 ug/g 1.7 ug/g 0.8 ug/g			Aas & Børseth, 2002
Cod (*Gadus morhua*)	Laboratory	1 ppm crude oil Statfjord B	14 days					Aas *et al*., 2002
Cod (*Gadus morhua*)	Laboratory	0.06 - 0.25 - 1 ppm Oil	average 3, 7, 14, 24 days	Naph type Pyren type BaP type		3.9 ug/g 2.6 ug/g 1.0 ug/g	7.5 – 23.7 – 31.4 3.6 – 10.6 – 13 1.7 – 2.4 – 2.2	Skadsheim *et al*., 2004
Cod (*Gadus morhua*)	Laboratory	0.06 - 0.25 - 1 ppm Oil	average 3, 17, 31 days	Naph type Pyren type BaP type		*53.1 ug/g* 7.0 ug/g 1.0 ug/g	0.7 - 2.3 - 2.9 1 - 2.9 - 3.3 1.1 - 1.5 - 1.5	Skadsheim *et al*., 2004

Species	Substance (lab/field)	Test concentrations/area	Exposure time	Metabolite	Baseline	Control or reference	exposed/control	reference/comments
Cod (*Gadus morhua*)	Laboratory	0.06 - 0.25 - 1 ppm Oil	30 days	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	Aas *et al*. 2000
Cod (*Gadus morhua*)	Laboratory	PW Oseberg, 1:1000 - 1:200 - 0.2 ppm oil - 0.2 ppm oil + PAH mix	15 days	Naph type Pyren type BaP type		12.6 ug/ml 4 ug/ml 1.8 ug/ml	1.3 - 2.5 - 3.6 - 5.4 1.7 - 3.7 - 4.1 -17.8 1.3 - 1.8 - 1.5 - 2.4	Sundt, 2004
Cod (*Gadus morhua*)	Field, Caged	North Sea - Statfjord, 10000 m - 2000m – 500 m German bight G	5.5 weeks	Naph type Pyren type BaP type	7.5 ug/ml 3.1 ug/ml 1.2 ug/ml	0.7 0.7 0.8	1.7 - 1.9 - 2.1 1.2 - 1.5 - 1.6 1.2 - 1.1 - 1.2	Aas *et al*., in press
Cod (*Gadus morhua*)	Field, Caged	Field, Caged German bight G4 (Ref) G1 - G2 - G3	5.5 weeks	Naph type Pyren type BaP type	7.5 ug/ml 3.1 ug/ml 1.2 ug/ml	0.4 0.5 0.7	0.9 - 0.9 - 1.6 0.8 - 0.9 - 1.7 0.8 - 1 - 1.3	Aas *et al*., in press
Cod (*Gadus morhua*)	Field, Caged	North Sea - Troll, 1000 m - 500m	6 weeks	Naph type Pyren type BaP type	4.6 ug/ml 2.4 ug/ml 0.9 ug/ml	1.4 0.9 1.1	1.7 - 2.5 1.1 - 1.3 1.1 - 1.3	Børseth *et al*., 2004
Cod (*Gadus morhua*)	Field, Caged	North Sea - Tampen, 10000 - 2500 – 1000 - 500	6 weeks	Naph type Pyren type BaP type		8.8 ug/ml 1.4 ug/ml	1.0 - 1.5 - 1.2 - 1.2 0.9 - 0.7 - 0.8 - 0.9	Hylland *et al*., 2005
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Egersund	Baseline non polluted area	Naph type Pyren type BaP type	5.1 ug/ml 1.4 ug/ml 0.7 ug/ml			Klungsøyr *et al*. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Sleipner	Baseline polluted area?	Naph type Pyren type BaP type	6.8 ug/ml 1.9 ug/ml 0.8 ug/ml			Klungsøyr *et al*. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Statfjord	Baseline polluted area?	Naph type Pyren type BaP type	11.2 ug/ml 2.5 ug/ml 0.7 ug/ml			Klungsøyr et al. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents Sea		Naph type Pyren type BaP type	2.52 ug/g 1.69 ug/g 0.77 ug/g			

Species	Substance (lab/field)	Test concentrations/area	Exposure time	Metabolite	Baseline	Control or reference	exposed/control	reference/comments
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents Sea		Naph type Pyren type BaP type	2.0 ug/g 1.3 ug/g 0.6 ug/g			Aas & Børseth, 2004
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Frøy, ceased installation 10 000 m (ref) 2000 m - 200 m	Baseline polluted area?	Naph type Pyren type BaP type		5.6 ug/ml 1.4 ug/ml 0.75 ug/ml	1.3 - 2.2 1.4 - 0.7 1.8 - 0.6	Beyer *et al*., 2003
Sheepshead minnow	Laboratory	North sea oil A 0.1 - 0.4 - 0.7 ppm	5 weeks	Naph type Pyren type BaP type		6916 569 107	2.3 - 6.2 - 9.3 2.5 - 5 - 6.3 4 - 13.1 - 19.2	Bechmann *et al*. 2004
Sheepshead minnow	Laboratory	North sea oil B 0.1 - 0.9 - 5.6 ppm	6 weeks	Naph type Pyren type BaP type		18164 438 110	1.8 - 4.3 - 12.5 5.6 - 12.6 - 30.8 12.6 - 42.7 - 123.9	Bechmann *et al*. 2004
Sheepshead minnow	Laboratory	2 - 14 - 214 ppb	5 weeks	Naph type Pyren type BaP type		267280 9926 5152.7	0.9 - 2.2 - 18.6 0.9 - 1.5 - 9.6 3 - 17.4 - 207	Bechmann *et al*. 2004
Polar cod (*Boreogadus saida*)	Laboratory, feral fish 2001, 2002	1.5 ppm Statfjord oil, baseline, control	14 days	Naph type Pyren type BaP type	16.0 ug/g 0.9 ug/g 0 ug/g	2 5.5 0	16.9 74.4 1.8	Sundt & Bechmann, 2004

Table 2. PAH-metabolites in marine fish measured by GC-MS.

Species	Substance (lab/field)	Test concentrations	Exposure time	Metabolite	Baseline	control or reference	exposed/control	Reference
Cod (*Gadus morhua*)	Feral fish	Barents sea	baseline	Naph sum Phen sum Pyren	150,6 ng/g 61,2 ng/g 4,6 ng/g			Aas & Børseth, 2002
Cod (*Gadus morhua*)	Feral fish	Barents sea	baseline	Naph sum Phen sum Pyren	1285 ng/g 220 ng/g 3.5 ng/g			Sundt, 2004
Cod (*Gadus morhua*)	Feral fish	Egersund	Baseline non polluted area	Naph sum Phen sum Pyren	2005.1 ng/g 230.2 ng/g 3.9 ng/g			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Sleipner	Baseline polluted area?	Naph sum Phen sum Pyren	1296.1 ng/g 197.8 ng/g 0 ng/g			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Feral fish	Statfjord	Baseline polluted area?	Naph sum Phen sum Pyren	1361.7 ng/g 351.1 ng/g 4.0 ng/g			Klungsøyr *et al*. 2003
Cod (*Gadus morhua*)	Laboratory	0.06 - 0.25 – 1 ppm Oil	average 3, 7, 14, 24 days	Naph sum Phen sum Pyren		2549 ng/g 691 ng/g 27 ng/g	4.6 - 13.4 - 23.6 7.7 - 22.9 - 34.9 7.3 - 16.2 - 25.1	Skadsheim *et al*., 2004
Cod (*Gadus morhua*)	Laboratory	0.06 - 0.25 – 1 ppm Oil	average 3, 17, 31 days	Naph sum Phen sum Pyren		5702 ng/g 377 ng/g 5 ng/g	4 - 13.3 - 12,7 10.5 – 40.3 – 48.7 8.6 - 63 – 88.4	Skadsheim *et al*., 2004
Cod (*Gadus morhua*)	Field, Caged	North Sea - Statfjord, 500 - 2000 - 10000 m		Naph sum Phen sum Pyren		1150 ng/g 340 ng/g	3.0 - 2.0 - 1.3 3.5 - 2.7 - 2.5	Aas *et al*., in press
Cod (*Gadus morhua*)	Field, Caged	North Sea - Troll, 1000 m - 500m	6 weeks	Naph sum Phen sum Pyren	1515.1 ng/g 327.2 ng/g 173.2 ng/g	1.1 1.6 1.2	1.1 - 1.2 2.1 - 2.0 0.9 - 1.2	Børseth *et al*., 2004

Species	Substance (lab/field)	Test concentrations	Exposure time	Metabolite	Baseline	control or reference	exposed/control	Reference
Cod (*Gadus morhua*)	Field, Caged	North Sea - Tampen, 10000 - 2500 - 1000 – 500	6 weeks	Naph sum Phen sum Pyren		965.3 ng/g 934.5 ng/g 3.7 ng/g	0.9 - 1.7 - 0.9 – 1 1.4 - 3 - 1.8 - 1.5 0 - 0 - 0.5 - 0.0	
Cod (*Gadus morhua*)	Field, Caged	North Sea - Statfjord, 10000 m - 2000m - 500 m	5.5 weeks	Naph sum Phen sum Pyren	228 ng/g 482 ng/g 28 ng/g	0.2 2.0 10.2	0.9 - 1.1 - 0.9 3 - 4.5 - 6.7 29.5 - 31.1 - 41.5	Aas *et al*., in press
Cod (*Gadus morhua*)	Field, Caged	German bight G4 (Ref) G1 - G2 - G3	5.5 weeks	Naph sum Phen sum Pyren	228 ng/g 482 ng/g 28 ng/g	0.8 1.0 0.0	1 - 1 - 1.9 0.7 - 0.8 - 0.8 0 - 0 - 0	Aas *et al*., in press
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Egersund	Baseline non polluted area	Naph sum Phen sum Pyren	1346.9 ng/g 526.8 ng/g 5.7 ng/g			Klungsøyr *et al*. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Sleipner	Baseline polluted area?	Naph sum Phen sum Pyren	1111.5 ng/g 331.5 ng/g 10.4 ng/g			Klungsøyr *et al*. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Statfjord	Baseline polluted area?	Naph sum Phen sum Pyren	1279.7 ng/g 331.9 ng/g 3.1 ng/g			Klungsøyr *et al*. 2003
Haddock (*Melanogrammus aeglefinus*)	Feral fish	Barents sea		Naph sum Phen sum Pyren	1474 ng/g 165 ng/g 0			
Polar cod (*Boreogadus saida*)	Laboratory, feral fish 2001, 2002	1.5 ppm Statfjord A oil, baseline, control	14 days	Naph sum Phen sum Pyren	1330 ng/g 538 ng/g 52 ng/g	1.3 0.9 14.6	114 90 60	

Table 3. Biological assessment criteria (BAC) and Environmental assessment criteria (EAC) for two PAH metabolites, different fish species and methods. Data partly taken from WKIMC 2009

Biological Effect	**Fish species**	**BAC [ng/ml] HPLC-F**	**EAC [ng/g] GC/MS**
Bile metabolite 1-hydroxypyrene	dab	16	
	cod	21	483
	flounder	16 ◊	
	haddock	13	
	dab, cod, haddock	17	
	turbot		909
	halibut		745
Bile metabolite 1-hydroxyphenanthrene	dab	3.7	
	cod	2.7	518
	flounder	3.7 ◊	
	haddock	0.8	
	dab, cod, haddock	2.4	
	turbot		1832
	halibut		262

Biological Effect	**Fish species**	**BAC [µg/ml] synchronous Fluor. 341/383**	**EAC [µg/ml] Fixed Fluor. 341/383**
Bile metabolites of pyrene-type	dab	0.15	22 *
	cod	1.1	35
	flounder	1.3	29 #
	haddock	1.9	35 ^
	turbot		29
	halibut		22
	herring/sprat		16

AC based on * halibut, # turbot, ^ cod and ◊ dab

Chapter 13: Reproductive success in eelpout (*Zoarces viviparus*)

Background

The eelpout (*Zoarces viviparus*), 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, PAH, 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 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 recommended by ICES, OSPAR and HELCOM for marine monitoring programmes of biological effects (OSPAR 1997, HELCOM 2006, ICES 2004), 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 guideline exists (Jacobsson *et al*., 1986; Neuman *et al*., 1999, Strand & Dahllöf, 2005). An international guideline is in preparation and to 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 contaminated areas 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 2). Acute larval mortality has also been observed in eelpout exposed to pulp mill effluents (Jacobsson *et al*. 1986). Other environmental stress factors like 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.

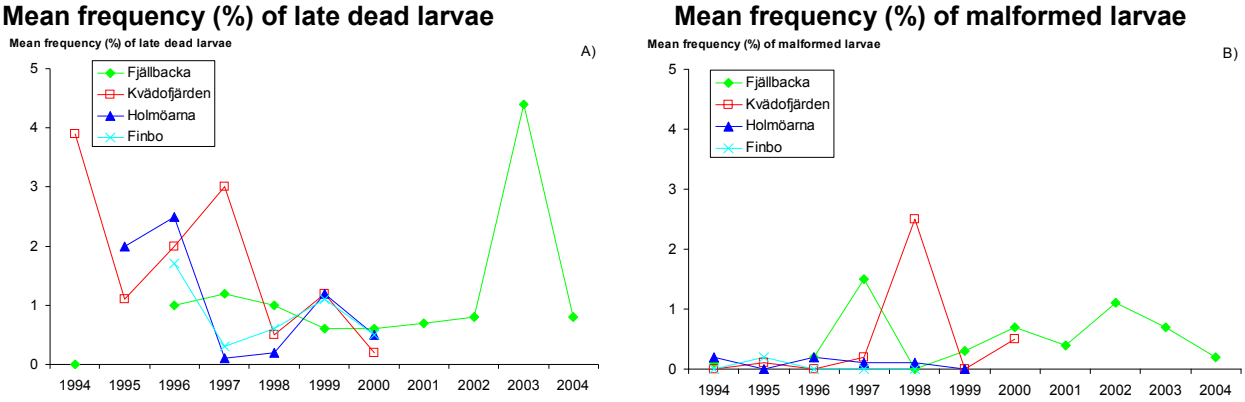


Figure 2. Year-to-year variations in mean frequencies of A) late dead larvae and B) malformed larvae at four Swedish monitoring stations regarded as reference sites

According to the technical guidelines used in the Swedish and Danish monitoring programmes (Neuman *et al*. 1999, Strand & Dahllöf 2005), supporting parameters like water temperature and salinity together with general fish physiological and reproductive parameters should be recorded when the 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 be seen as supplementary parameters and how they can be integrated 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 & Dahllöf 2005), be characterised as;

- 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.

Similar with studies on skewed sex ratio in eelpout broods, although it 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 & Förlin 2002). In eelpout broods the reference conditions are supposed to be 50:50 between females and males.

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 significant different from the background response, i.e. where the impact of environmental factors such as contaminants can be regarded as close to zero.

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 compared to the North Sea and the North Atlantic.

Both data related to frequencies (mean percents) of abnormal larvae per female and frequencies 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 was found available of broods with >5% growth retarded larvae.

Data on frequencies of females with (at least one) abnormal larvae present in the brood is 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 90% percentile of all datasets observed in areas regarded as reference sites, i.e. in distance to larger cities and industry.

Assessment criteria related to mean frequencies of abnormal larvae in broods

Most studies on development of eelpout embryo and larvae from the Baltic Sea, the Kattegat and the Skagerrak studies 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 which were considered as reference sites only small frequencies of abnormal larvae have been found, if any. Values of 90% percentiles have been found to be 1% malformed larvae, 2% late dead larvae and 4% growth retarded larvae, respectively.

Table 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 90% percentile of response at reference sites	Elevated effect levels

Comparisons of datasets shows that class II, i.e. elevated mean frequencies of malformed larvae and late dead larvae, mainly have be found in areas which are not regarded as reference sites, i.e. suspected to be

more polluted (Figure 3). However, only one of the datasets shows significantly elevated levels of growth retarded larvae in the broods.

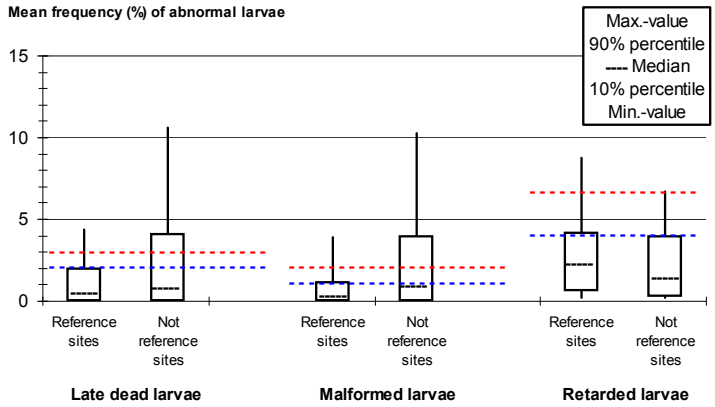


Figure 3. Comparison of data distribution of data on mean frequencies of late dead, malformed and growth retarded larvae in eelpout broods from reference sites and area not regarded as reference sites. The blue dotted line refers to the 90% percentile of data from the reference sites. The red dotted line refers to significantly elevated levels compared to the 90% percentile of the reference sites.

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 studies 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 (90% percentiles: 5%), of the pregnant eelpout containing elevated frequencies of late dead and malformed larvae in the broods (i.e. >5%).

Table 2. Proposed assessment criteria for the frequencies of pregnant eelpouts, which contain more than 5% malformed larvae and late dead larvae in their broods.

Assessment class	**Class I** **Background response**	**Class II**
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 90% 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 4).

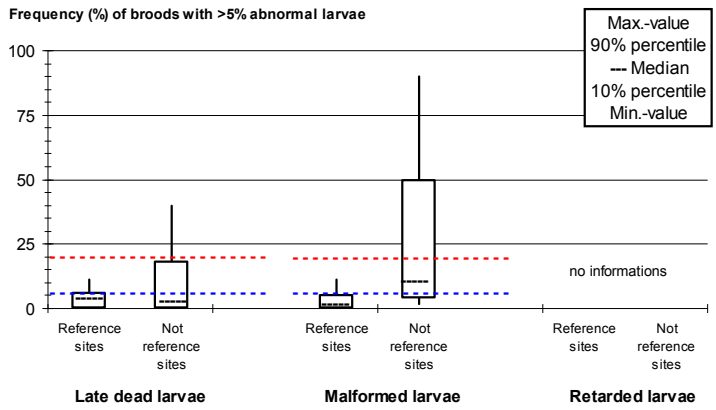


Figure 4. Comparison of data distribution of data on frequencies of broods containing >5% late dead larvae and malformed larvae in from reference sites and area not regarded as reference sites. The blue dotted line refers to the 90% percentile of data from the reference sites. The red dotted line refers to significantly elevated levels compared to the 90% percentile of the reference sites.

The assessment criteria including the existing data material and the statistical analyses will be evaluated and updated by 2011 as part of a BONUS+-project, called Balcofish.

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, since 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 90% 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.

References

Ådjers, K., Appelberg, M., Eschbaum, R., Lappalainen, A., Lozys, L. 2001. Coastal fish monitoring in Baltic reference areas 2000. Kala- ja riistaraportteja 229: 1-14, Helsinki, Finland. Agenda item 3 at the HELCOM MONAS meeting 22 - 24 March 2004 in Tallinn, Estonia. www.helcom.fi

BEQUALM 2000. Fish Reproductive Success embryo development and survival in viviparous blenny *Zoarces viviparus*. Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM). BEQUALM Newsletter 2000. http://www.uni-kiel.de/ftzwest/downloads/BQ-Newsletter2.pdf

Blekingekustens Vattenvårdsförbund 2005. Hanöbugten 2000-2002. http://www.bkvf.org/huvudsida.htm (In Swedish).

Bodammer, J.E. 1993. The teratological and pathological effects of contaminants on embryonic and larvae fishes exposed as embryos: a brief review. Am. Fish Soc. Symp. 14: 77-84.

Davis, W.P. 1997. Evidence for developmental and skeletal responses as potential signals of endocrine disrupting compounds in fishes. *In* Chemically induced alterations in functional development and reproduction of fishes. Edited by Rolland, R.M., Gilbertson, M., Peterson, SETAC Press, Pensacola FL.

Fagerholm, B. 2002. Integrerad fiskövervakning i kustreferensområden, Fjällbacka. Årsrapport för 2001. Fiskeriverket, Kustlaboratoriet. Arbetsrapport. 10p. (In Swedish).

Gercken, J., Förlin, L., Andersson, J. 2006. Developmental disorders in larvae of eelpout (*Zoarces viviparus*) from German and Swedish Baltic coastal waters. Mar Pollut Bull. 53(8-9):497-507.

HELCOM 2006. Manual for Marine Monitoring in the COMBINE Programme of HELCOM. Part D. Programme for monitoring of contaminants and their effects. http://www.helcom.fi/groups/monas/CombineManual/en_GB/Contents/, updated 31.03.2006

ICES 2004. Recommended techniques for biological monitoring programmes at the national or international level methods for fish. Report of the Working Group on Biological Effects of Contaminants. WGBEC meeting 22 - 26 March 2004 Ostend, Belgium. www.ices.dk/reports

Jacobsson, A., Neuman, E., Thoresson, G. 1986. The viviparous blenny as an indicator of environmental effects of harmful substances. Ambio 15(4): 236-238.

Kalmarweb, 2005. Samordnad kustvattenkontroll i Kalmar län. www.smhi.se/miljo/kalmarweb (In Swedish).

Larsson, D.G.J, Forlin, L. 2002. Male-biased sex ratios of fish embryos near a pulp mill: Temporary recovery after a short-term shutdown. Environmental Health Perspective 110(8): 739-742.

Neuman, E., Sandström, O., Thoresson, G. 1999. Guidelines for coastal fish monitoring. National Board of Fisheries, Institute of Coastal Research, Ôregrund, Sweden, 44p

OSPAR 1997. JAMP guidelines for general biological effects monitoring. Technical Annex 10 reproductive success in fish. www.ospar.org

Sjölin, A., Olsson, P., Magnusson, G. 2003. Hälsotillstånd och fortplantning hos tånglake i recipienten til Mörrums bruk hösten 2002. Toxicon rapport 8/03. http://www.bkvf.org/Rapporter/Rapporter/Tånglake/2003/toxicon.pdf (In Swedish)

Strand, J., Andersen, L., Dahllöf, I., Korsgaard, B. 2004. Impaired larval development in broods of eelpout (*Zoarces viviparus*) in Danish coastal waters. Fish Physiology and Biochemistry 30: 37 46

Strand, J, Dahllöf, I. 2005. Biologisk effektmonitering i fisk. NOVANA teknisk anvisning for marin overvågning, kapitel 6.3. Danmarks Miljøundersøgelser. http://www2.dmu.dk/1_Om_DMU/2_Tvaerfunk/3_fdc_mar/programgrundlag/tekanv/tekniskanv.asp (In Danish).

Strand, J. 2005a. Biologisk effektmonitering i havsnegle og fisk. Marine workshopper 2004. Research Note from NERI No. 210, 34p. http://www.dmu.dk/Udgivelser/Arbejdsrapporter (In Danish).

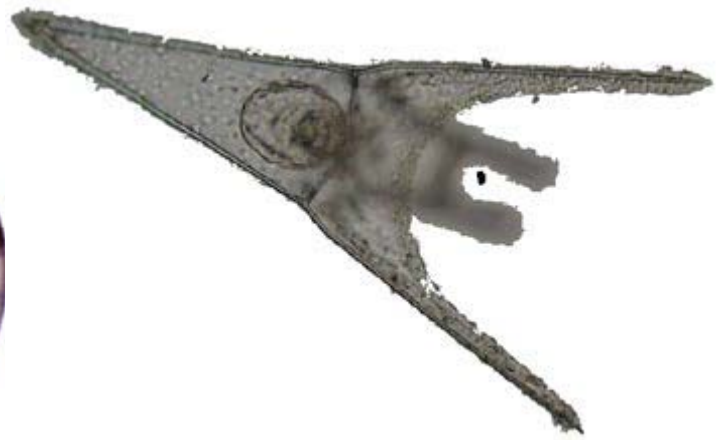
Strand, J. 2005b. Bioligiske effekter i ålekvabbe og muslinger. Marine områder 2004. Status og trends i NOVANA overvågning. Faglig rapport fra DMU. www.dmu.dk. (In Danish).

Vetemaa, M. 1999. Reproduction biology of the viviparous blenny (*Zoarces viviparus* L.). Rep. Nat. Board Fish. Swed. 2: 81-96, Fiskeriverket, Göteborg, Sweden.

Vetemaa, M., Förlin, L., Sandström, O. 1997. Chemical effluent impacts on reproduction and biochemistry in a North Sea population of viviparous blenny (*Zoarces viviparus*). J. Aquat. Ecosys. Stress Rec. 6: 33-41.

Chapter 14: Sediment seawater elutriate and pore-water bioassays with early developmental stages of marine invertebrates Bivalve D-larva Sea-urchin *pluteus* larva

Bivalve D-larva



Sea-urchin pluteus larva

Background

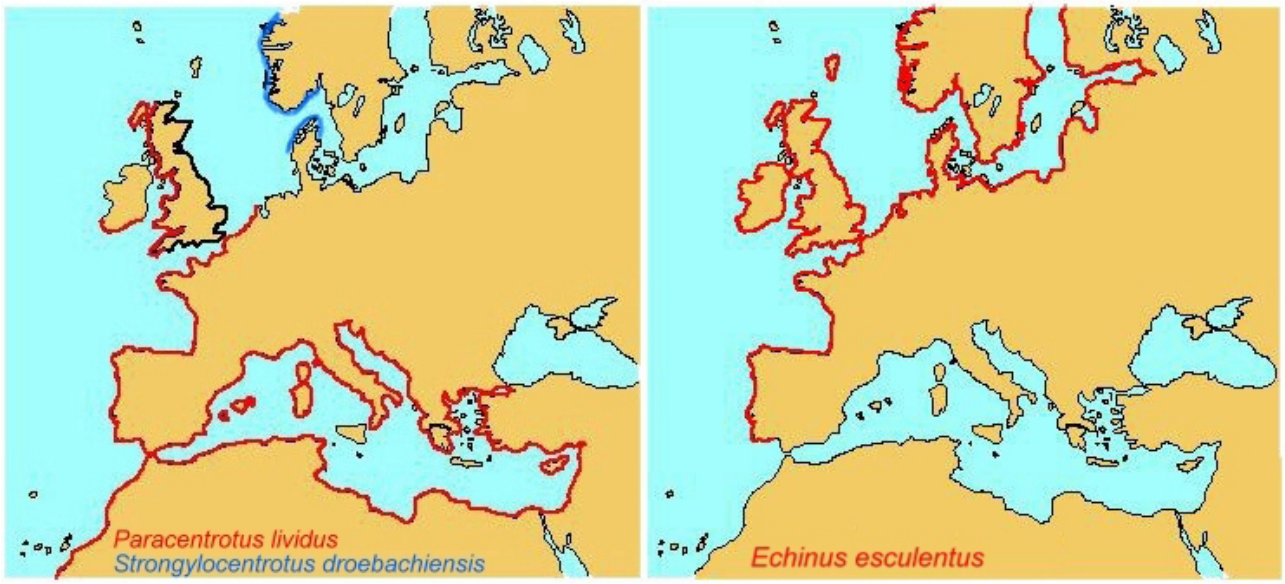
The embryogenesis and early larval development of marine invertebrates have been frequently used as a rapid, sensitive, cost-effective biological tool for the assessment of seawater, sediment elutriates and pore-water quality. Early developmental stages are generally more sensitive than adults and the 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 µg/L for TBT and other antifouling products, 10 µg/L for Hg, Cu and Zn, 100 µg/L for Pb, Cd and other metals, 1 mg/L for organochlorine pesticides, detergents and refined oil, and 10 mg/L 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 to 48 hours incubation at 18 to 24ºC (depending on the species), samples are fixed and microscopically observed to record the percentage of normally developed larvae and, in the case of 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 EC50 values of 18 priority pollutants to bivalve *vs*. sea-urchin embryos reflected a correlation coefficient r2=0.96 ($p<0.01$) and a slope b=1.00 (Beiras and Bellas, 2008). Due to their abundance and broad geographical distribution or availability from commercial sources the following species are recommended: *Crassostrea gigas*, *Mytilus edulis/galloprovincialis*, *Paracentrotus lividus*. In the case of sea-urchins, other species like *Strongylocentrotus droebachiensis* and *Echinus sculentus*, extend the applicability of the assay with indigenous species to Northern countries (see figures).

Within bivalves, *Crassostrea gigas* and, in the U.S. C. *virginica* oysters have been most often used for embryo-larval ecotoxicological bioassays because, unlike the mussel or the native flat oyster (*Ostrea edulis*), in *Crassostrea* fertile gametes 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*., 2000). The marine mussels of the *Mytilus genus* occurring in European waters (*M. edulis* and *M.*

galloprovincialis) are nearly ubiquitous, easy to collect and to maintain in aquaria. Also these species show 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 to 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 avoiding accidental spawning. Another advantage of the sea-urchin embryogenesis bioassay is to provide a quantitative, more gradual, observer-independent and statistically treatable response: size increase (Saco-Álvarez *et al*., 2010).



Currently, the main limitation of the embryo-larval bioassays is the availability of reliable, good quality biological material all 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 all year round. Cryopreservation of gametes of bivalves and sea-urchins is a promising solution to provide homogeneous biological material at any time, but up to date these techniques are still on development and standard methods are not available. Combination of different species with different spawning seasons seems to be still 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: smaller amounts of sediment and simpler equipment are necessary, the environmental parameters of the elutriate (dissolved oxygen, pH, salinity, ammonia, sulphides) are closer to those of the natural water column than in the case of 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 sea-water 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, since when the confounding factors are taken into account both methods yield comparable results (Beiras, 2001).

Generally, 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). However, 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.

Confounding factors

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

The presence of the toxic substances such as unionized ammonia and H2S 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; Matthiesen *et al*., 1998). Some threshold toxicity values for sea-urchin and bivalve embryos are available in the literature (Knezovic *et al*., 1996), but further research is strongly needed on this topic. For NH3 Saco-Álvarez *et al*. (2010) obtained an EC10 of 68.4 µg/L and a NOEC/LOEC of 40/80 µg/L using *Paracentrotus lividus*.

Regarding 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, 20ºC (48 h) is recommended for mussels and *Paracentrotus lividus* urchins, and 24ºC (24 h) for *Crassostrea gigas* oysters.

Ecological relevance

The 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.

Assessment criteria

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

End-points measured

The end-point recorded in the standard embryo-larval bioassays is the percentage of morphologically normal larvae.

The definition of morphological abnormalities change among authors and, obviously, among test species. For routine applicability's sake it is advised that only very conspicuous abnormalities were 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 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. Pre-*pluteus* stages where oral arms were not yet fully separated, or larvae with missing arms, should be considered as abnormal. However more detailed abnormalities such as those related to the internal anatomy of the larvae (skeletal rods, gut) would greatly complicate observation. Their identification even depends on the position of the larva under the microscope.

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

Assessment criteria

Discrete approach: ICES (2008) 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 to 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 we were sure from comprehensive analytical data that it is not polluted but was otherwise similar to the problem sites (see confounding factors). Control seawater may not be appropriate as reference because it lacks the physicochemical and microbiological properties of an elutriate, some of which may affect the response.

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). TU = 100 / ED50, where ED50 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 were pooled together for statistical analysis, they must be previously corrected by the respective controls by using Abbott's formula: P'=(P-Pc)x100/(100-Pc); where P and P' are the raw and corrected abnormality percentages, and Pc 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).

In the case of 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 to reference. The methodology to obtain BAC and EAC values followed OSPAR (2009). The resulting BAC value was PNR=0.702, which means a 30% decrease in growth (size increase) in the tested population.

A more detailed evaluation of the results from the sea-urchin test can be obtained by pooling the results from sites not significantly different to reference in a first dataset, and pooling toxic sites in a second dataset. Taking different percentiles from those distributions the following environmental assessment criteria (EAC) for Percent Net Response (PNR) and Toxic Units (TU) data were obtained.

A BAC of 22 was set for mussel larvae (Table 1).

EAC-values for both assays were retained at 50% as recommended earlier by ICES, either mortality (mussel embryo) or reduced growth (sea urchin embryo).

Table 1. Background response for mussel embryo bioassays (mortality); data from IEO-Vigo.

Average	90-percentile	median	10-percentile	N
14.7	22.3	8	3.2	38

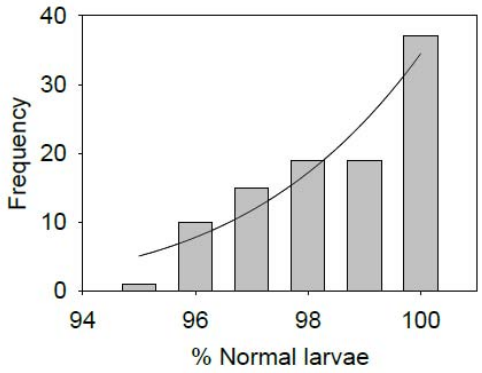
Quality assurance

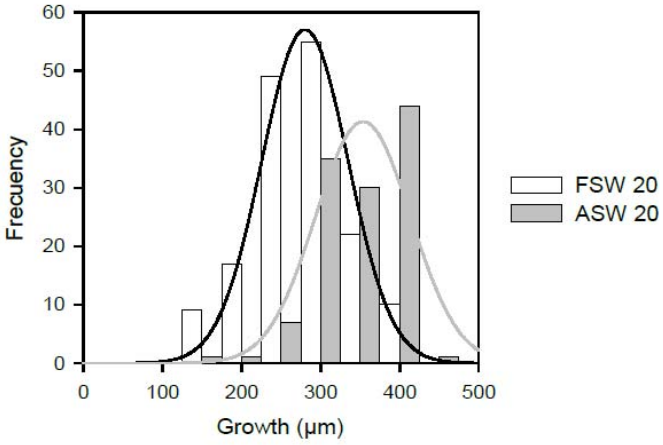
Sediment manipulations during sampling, storage and testing, and quality of the test organisms have been often identified as the main sources of variability in sediment toxicity bioassays. Concerning the first point, sediments intended to toxicity testing should not be frozen but stored under refrigeration 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 porewater) after elimination of particles. However it must be taken into account that glass fibre filters adsorb metals and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken and 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 interpopulation variability. In the case of 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 1 below. 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.

In the case of the *P. lividus* normal larval development, the distribution of the endpoints measured (percentage of normal larvae, and size increase) in controls with natural filtered seawater (FSW) and artificial seawater (ASW), throughout several years of tests conducted at 20ºC for 48h, was the following (Saco-Álvarez *et al*., 2010):





From these data, and taking the 5th 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 (natural filtered seawater) or 253 µm in ASW (artificial seawater).

Percentage fertilization prior to testing must always be recorded. To run a reference toxicant test may be further useful to check the biological quality of the test organisms using a chart of the reference toxicant EC50 historical values.

References

AFNOR, 2009. Qualité de l'eau. Bioindicateur de la toxicité potentielle de milieux aqueux. Détermination de la toxicité potentielle d'échantillons aqueux sur le développement embryo-larvaire de bivalve. Norme expérimentale XP T90-382. Septembre 2009. AFNOR.

ASTM, 1995. Standard guide for conducting static acute toxicity tests with echinoid embryos. ASTM. (E 1563-95), 962–980. Annual Book of ASTM Standards. American Society for Testing and Materials.

Becker, D.S., Bilyard, G.R., Ginn, T.C., 1990. Comparisons between sediment bioassay and alterations of benthic macroinvertebrate assemblages at a marine superfund site: Commencement Bay, Washington. Environmental Toxicology and chemistry 9:669–685.

Beiras, R., 2001. Comparison of methods to obtain a liquid phase in marine sediment toxicity bioassays with *Paracentrotus lividus* sea urchin embryos. Arch. Environ. Contam. Toxicol. 42, 23–28.

Beiras, R., Saco-Álvarez, L., Fernández, N., González, J.J., 2005. Bivalve and sea-urchin embryogenesis bioassay as biological tool for monitoring coastal pollution in Europe. SETAC Europe 15th annual meeting, Lille.

Beiras, R., Bellas, J., 2008. Inhibition of embryo development of the *Mytilus galloprovincialis* marine mussel by organic pollutants; assessment of risk for its extensive culture in the Galician Rias. Aquaculture 277:208–212.

Cardwell, R.D., Woelke, C.E., Carr, M.I., Sanborn, E.W., 1976. Sediment and elutriate toxicity to oyster larvae. In: Proceedings of the speciality conference on Dredging and its environmental effects. Mobice, Alabama. American society of Civil Engineers, New York.

Carr, R.S., Chapman, D.C., 1992. Comparison of solid-phase and pore-water approaches for assessing the quality of marine and estuarine sediments. Chemistry and Ecology. 7:19–30.

Carr, R.S. 1998. Marine and estuarine porewater toxicity testing. In: Wells PG, Lee K, Blaise CB (eds) Microscale testing in aquatic toxicology. Advances, techniques, and practice. CRC Press, Boca Raton, p 523–538.

Durán, I., Beiras, R., 2010. Assessment criteria for using the Sea-urchin Embryo Test with sediment elutriates as a tool to classify the Ecotoxicological status of marine water bodies. Environ. Toxicol. Chem. 29: 1192–1198.

His, E., Beiras, R., 1995. Monitoring fresh and brackish water quality around shellfish farming areas with a bivalve embryo and larva simplified bioassay method. Oceanologica Acta, 18, 591–595.

His, E., Seaman, M.N.L., Beiras, R., 1997. A simplification of the bivalve embryogenesis and larval development bioassay method for water quality assessment. Wat. Res. 31(2): 351–355.

His, E., Beiras, R., Seaman, M.N.L., 1999. The assessment of marine pollution - Bioassays with bivalve embryos and larvae. In: Southward, A.J., Tyler, P.A., Young, C.M. (Eds.) Advances in Marine Biology Vol. 37, Academic Press, San Diego, pp. 1–178.

ICES, 2008. Report of the Fourth ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON IV). ICES CM2008/ACOM 49. ICES Advisory Committee. Copenhagen, 71 pp.

Knezovich, J.P., Steichen, D.J., Jelinski, J.A., Anderson, S.L., 1996. Sulfide tolerance of four marine species used to evaluate sediment and pore-water toxicity. Bull Environ Contam Toxicol 57, 450–457.

Kobayashi, N., 1995. Bioassay data for marine pollution using echinoderms. In: Cheremisinoff PN (ed) Encyclopedia of environmental control technology. Gulf Publ. Co., Houston, p 539–609.

Long, E.R., Buchman, M.F., Bay, S.M., Breteler, R.J., Carr, R.S., Chapman, P.M., Hose, J.E., Lissner, A.L., Scott, J., Wolfe, D.A., 1990. Comparative evaluation of five toxicity tests with sediments from San Francisco Bay and Tomales Bay, California. Environmental Toxicology and chemistry 9:1193–1214.

Long, E.R., Robertson, A., Wolfe, D.A., Hameedi, J., Sloane, G.M., 1996. Estimates of the spatial extent of sediment toxicity in major U.S. estuaries. Environmental Science and Technology 30:3585–3592.

Matthiessen, P., Bifield, S., Jarret, F., Kirby, M.F., Law, R.J., McMinn, W.R., Sheahan, D.A., Thain, J.E., Whale, G.F., 1998. An assessment of sediment toxicity in the river Tyne estuary, UK by means of bioassays. Marine Environmental Research 45:1–15.

OSPAR, 2009. Agreement on CEMP assessment criteria for the QSR2010, Agreement number: 2009-2. OSPAR Commission.

Quiniou, F., His, E., Delesmont, R., Caisey, X., 2005. Bio-indicateur de la toxicité potentielle de milieux aqueux: bio-essei “developpement embryo-larvaire de bivalve”. Ed. IFREMER, 24 pp.

Saco-Álvarez, L., Durán, I., Lorenzo, J.I., Beiras, R. 2010. Methodological basis for the optimization of a marine sea-urchin embryo test (SET) for the ecological assessment of coastal water quality. Ecotoxicol. Environ. Saf. 73: 491–499.

Thain, J.E., 1991. Biological effects of contaminants: oyster (*Crassostrea gigas*) embryo bioassay. ICES. (No. 11). 1991. Copenhagen, ICES. Techniques in marine environmental sciences.

Williams, L.G., Chapman, P.M., Ginn, T.C., 1986. A comparative evaluation of marine sediment toxicity using bacteria luminescence, oyster embryo and amphipod sediment bioassays. Marine Environmental Research 19:225–249.

Chapter 15: Sediment seawater elutriate and pore-water bioassays with copepods (*Tisbe, Acartia*), mysids (*Siriella, Praunus*), and decapod larvae (*Palaemon*)

Tisbe battagliai

Siriella armata

Palaemon elegans

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 to other marine invertebrates. Therefore when these contaminants were 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*., 1998). Detailed methods are available (Hutchinson and Williams, 1989; UNEP, 1989). The endpoint recorded may be mortality or motility after 48 to 96h incubation in the test samples at 20ºC 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 test organisms by US-EPA for estuarine and marine water toxicity tests (US-EPA 2002). The maintenance of fertile adult stocks in aquaria, fed on *Artemia*, is feasible. Since these organisms undergo direct development in short time periods they are suitable for life cycle assessments. Some European mysids such as *Neomysis* (for brakish waters), *Praunus* (Garnacho *et al*., 2000; Mclusky and Hagerman, 1987) 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 decapods 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 to obtain ovigerous females from commercial stocks. The main restriction is to find broadly distributed species across all Europe. The *Palaemon* genus may be a potential candidate since it shows a broad geographical distribution, from Mediterranean Sea to North Sea, they are easy to feed, the maintenance of fertile adult stocks in aquaria is feasible, and larval development is well known.

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 testing and they must fall within optimum ranges for the survival and motility of the test species or otherwise adjusted. This is particularly important in the case of pore waters from highly reduced sediments, which broadly depart from those values.

More often the presence of toxic reduced compounds, un-ionized ammonia and H2S, have been identified as the main sources of false positives in sediment elutriate toxicity testing (Cheung *et al*., 1997). Further research is strongly needed on this topic.

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 food web in coastal and oceanic ecosystems.

Assessment criteria

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

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 to toxicity testing should not be frozen but 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 porewater) after elimination of particles. However it must be taken into account that glass fibre filters adsorb metals and some organic filters might retain organic compounds, so refrigerated centrifugation may be preferred. After thawing, samples should be shaken and 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 1, resulting in a BAC of 5.0.

Table 1. Preliminary results of background response levels for *Tisbe* bioassays (mortality) – data from Cefas.

Average	**210-percentile**	**median**	**90-percentile**	**N**
1.3	0.0	0.0	5.0	28

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

References

Bellas, J., Beiras, R., Mariño-Balsa, J.C., Fernández, N., 2005. Toxicity of organic compounds to marine invertebrate embryos and larvae: a comparison between the sea urchin embryogenesis bioassay and alternative test species. Ecotoxicology, 14: 337–353.

Cheung, Y.H., Neller, A., Chu, K.H., Tam, N.F.Y., Wong, C.K., Wong, Y.S., Wong, M.H., 1997. Assessment of sediment toxicity using different trophic organisms. Arch. Environ. Contam. Toxicol. 32: 260–267.

Garnacho, E., Peck, L.S., Tyler, P.A.A., 2000. Variations between winter and summer in the toxicity of copper to a population of the mysid *Praunus flexuosus*. Mar Biol 137:631–636.

Hutchinson, T.H., Williams, T.D., 1989. The use of sheepshead minnow (*Cyprinodon variegatus*) and a benthic copepod (*Tisbe battagliai*) in short-term tests for estimating the chronic toxicity of industrial effluents.

ICES, 2008. Report of the Fourth ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON IV). ICES CM2008/ACOM 49. ICES Advisory Committee. Copenhagen, 71 pp.

Mariño-Balsa, J.C., Poza, E., Vázquez, E., Beiras, R., 2000. Comparative toxicity of dissolved metals to early larval stages of *Palaemon serratus*, *Maja squinado* and *Homarus gammarus* (Crustacea: Decapoda). Arch. Environ. Contam. Toxicol. 39, 345–351.

Matthiessen, P., Bifield, S., Jarret, F., Kirby, M.F., Law, R.J., McMinn, W.R., Sheahan, D.A., Thain, J.E., Whale, G.F., 1998. An assessment of sediment toxicity in the river Tyne estuary, UK by means of bioassays. Marine Environmental Research 45:1–15.

Mclusky, D.H., Hagerman, L., 1987. The toxicity of chromium, nickel and zinc: effects of salinity and temperature, and the osmoregulatory consequences in the mysid *Praunus flexuosus*. Aquat Toxicol 10:225–238.

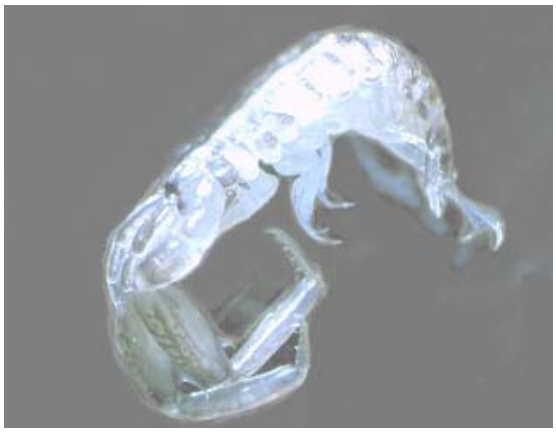
Pérez, S., Beiras, R., 2010. The mysid *Siriella armata* as a model organism in marine ecotoxicology: comparative acute toxicity sensitivity with *Daphnia magna*. Ecotoxicology 19:196–206.

Ramamoorthy, S. and Baddaloo, E.G., 1995. Handbook of chemical toxicity profiles of biological species Vol I Aquatic species, Lewis Publishers, Boca Raton.

UNEP, FAO, and IAEA. Estimation of the toxicity of pollutants to marine phytoplanktonic and zooplanktonic organisms. (44), -24. 1989. Reference methods for marine pollution studies.

US-EPA, 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to marine and estuarine organisms. EPA-821-R-02-014. US Environmental Protection Agency, Washington.

Chapter 16: Whole sediment bioassays with amphipods (*Corophium spp*) and *Arenicola marina*



Corophium multisetosum

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 ten days incubation in the whole sediment at 20ºC. These protocols can be easily adapted to the European species (*Corophium* spp). Some efforts have already been made to compare methods and sensitivity 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 ICES claims that 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 be also suitable after methodological standardisation and sensitivity comparisons with amphipods. Furthermore, *Corophium* is not tolerant to coarse grain sediments. Should sandy sediments be tested alternative species such as *Arenicola*, *Echinocardium* or *Cerastoderma* will be needed.

Some sublethal responses have been proposed as additional endpoints in order to enhance sensitivity, including reburial after the ten day exposure (Bat and Raffaelli, 1998), and 28-day 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.

Confounding factors

The presence of toxic reduced compounds such as un-ionized ammonia and H2S in interstitial and overlying water has been identified as confounding factors in whole sediment toxicity testing (Phillips *et al*., 1997). The studies have been carried out with North America species. Further research on this topic with *Corophium* spp. is strongly needed.

Grain size also affects amphipod survival (De Witt *et al*., 1988). The studies have been carried out with North America species. Further research on this topic with *Corophium* spp. is strongly needed.

Assessment criteria

According to 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 (2008) 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, 2008).

ANOVA and *a posteriori* Dunnett's test allows comparison to control 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 by using Abbott's formula: P'=(P-Pc)x100/(100-Pc); where P and P' are the raw and corrected mortality percentages, and Pc is the control mortality. In this case no control treatment is available and Tukey's rather than Dunnett's posthoc test is preferred. Again, mortality data must be arcsine transformed prior to analysis.

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 to toxicity testing should not be frozen but stored under refrigeration in the dark inside airtight containers, and tested within one week.

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

Acceptability criteria must be developed concerning 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 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. 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) raise this threshold to 15%. Results of background response levels for *Corophium* and *Arenicola* bioassays are shown in Table 1. All laboratories show a 90th percentile for mortality higher than 10% and most above the recommended 15%, indicating that special care must be taken in avoiding any damage to the individuals during collection, maintenance and transfer into the experimental beakers.

The third year of a bioassay programme is running within BEQUALM from December 2006 to June 2007, and includes the 10-d *Corophium volutator* survival bioassay.

Table 1 Background response levels for whole sediment bioassays (mortality); the median 90-percentile, i.e. BAC, is 18.4%.

Test	lab	Average	10-percentile	median	90-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
Test	**lab**	**Average**	**10-percentile**	**median**	**90-percentile**	**N**
Arenicola	Cefas	4.7	0.0	0.0	13.3	20

References

ASTM. Standard guide for conducting 10-day static sediment toxicity tests with marine and estuarine amphipods. ASTM. (E 1367-92), 1138–1163. 1992.

Bat, L., Raffaelli D. 1998. Sediment toxicity testing: a bioassay approach using the amphipod *Corophium volutator* and the *polychaete Arenicola marina*. J Exp Mar Biol Ecol 226: 217–239.

Casado-Martínez, M.C., Beiras, R., Belzunce, M.J., González-Castromil, M.A., Marín-Guirao, L., Postma, J.F., Riba, I., DelValls, T.A. 2006. Interlaboratory assessment of marine bioassays to evaluate the environmental quality of coastal sediments in Spain. IV. Whole sediment toxicity test using crustacean amphipods. Ciencias Marinas 32(1B).

De Witt, T.H., Ditsworth, G.R., Swartz, R.C. 1988. Effects of natural sediment features on survival of the phoxocephalid amphipod *Rhepoxynius abronius* Mar Environ Res 25: 99–124.

ICES, 2008. Report of the Fourth ICES/OSPAR Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON IV). ICES CM2008/ACOM 49. ICES Advisory Committee. Copenhagen, 71 pp.

OSPAR, 1995. PARCOM Protocols on methods for the testing of chemicals used in the offshore oil industry.

Phillips, B.M., Anderson, B.S., Hunt, J.W. 1997. Measurement and distribution of interstitial and overlying water ammonia and hydrogen sulfide in sediment toxicity tests. Mar Environ Res 44(2): 117–126.

Swartz, R.C., de Ben, W.A., Jones, J.K.P., Lamberson, J.O., Cole, F.A. 1985. Phoxocephalid amphipod bioassay for marine sediment toxicity. In: Aquatic toxicology Hazard Assessment: 7th Symposium, ASTM STP 854, pp. 284–307. Ed. by RD Cardwell, R Purdy and RC Bahner. American Society for Testing Materials, Philadelphia.

Roddie, B., Thain, J. 2001. Biological effects of contaminants: *Corophium* sp. sediment bioassay and toxicity test. ICES Techniques in Marine Environmental Sciences, No. 29, 21 pp.

US EPA, 1994. Methods for assessing the toxicity of sediment-associated contaminants with estuarine and marine amphipods. United States Environmental Protection Agency, EPA/600/R-94/025.

US EPA, 1998. Evaluation of dredge material proposed for discharge in waters of the US. Testing manual. United States Environmental Protection Agency, EPA/823/F/98/005.

Chapter 17: Water *in vivo* bioassays

Executive summary

Applicability across the OSPAR maritime area: 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. The preferred method is short-term tests on concentrates of water. This includes both 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 fresh water, allowing all types of water to be assessed in the same way, and 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 of 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. A protocol for extraction methods for bioassays will become available as ICES TIMES series document in 2011.

Influence of environmental variables: Abiotic testing conditions, such as temperature, salinity, solids and especially 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; a background response, a warning level and 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 two times the background response and 100% mortality, and the warning level between these values.

In this document, 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 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 is 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.

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 fresh water whole effluents (e.g. Oris and Klaine, 2000; Power, 2004), and for dredged material.

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; Matthiesen *et al*., 1993; Hendriks et al., 1994; Thomas *et al*., 1999; Kirby *et al*., 1998; Peters *et al*., 2002; Akerman 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. 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 micro surface layer monitoring (Stebbing *et al*., 1992; Hylland et al., 2002, 2006).

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 on national level by several countries (ICES, 2004). The uptake of water bioassays, such as the oyster embryo assay (Thain *et al*., 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 micro-organisms) are now also frequently used as tools in estimating the potential risk of contaminants of estuarine and marine waters (e.g. Thomas *et al*., 2002; Akerman *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 sub cellular fractions isolated from organisms or modified bacteria. These tests are mechanism-based. They are 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 as to whether 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 fresh water 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, that they produce rapid results, that a smaller sample volume is needed and that 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, therefore, 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.

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 intra laboratory 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 idea being that 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 enable all possible substances to be covered, at both the acute and the chronic level. The set should therefore also have the following qualities:

Ecologically and/or toxicologically relevant

Relevance refers to the guarantee that the bioassay will measure the toxic and ecological effect one is actually interested in. Relevance is determined, among other things, by the test's sensitivity, specificity and discriminatory capacity. Ideally the measured effect should be ecologically relevant and if it is a species that is of ecological/commercial importance then 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 a weight of evidence as to the ecological relevance of the observed effects.

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, a set of at least three or four *in vivo* bioassays would be needed, one of which used fish.

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 the contaminants that are present in a test sample (i.e. tests lack specificity but have high relevance). At the moment, there is no one *in vivo* bioassay that could be used to detect all possible mechanisms of toxicity and indeed 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 also 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.

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, targeted bioassay use 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.

Reliable and reproducible

The reliability or precision of a bioassay relies on its reproducibility within the same laboratory, or in other laboratories (intra- and inter-laboratory 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.

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 can either 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:

Financial

In general, bioassays are not expensive (relative to other methodologies) and their incorporation into the CEMP should not entail excessive cost. However it is not possible to specify any particular sum, but 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.

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 micro-scale in operation, which by definition require less space and are more cost-effective.

Use of test animals

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

Availability of test 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.

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. Working with concentrates has a number of important advantages:

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, fresh water, 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 Convention 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 estrogenic effect.

Bioassays conducted on surface water samples generally use a small sample volume, typically 20–100ml taken from a discrete water sample of say two 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 up by bioassay led TIE (Toxicity Identification Evaluation; US EPA 1991) 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 are extracted and the efficiency of the extraction process will depend on the medium and solvent used. Metals, in particular, tend to get left behind 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 micro pollutants. The current extraction methods would appear to be broad enough for organic micro pollutants. 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 2005.

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 ecological 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.

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) 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).

b) A recommended method (or combination of methods) must have been shown to respond to contaminant exposure in the field.

c) 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 and include the methods turbot juvenile acute, *Daphnia* acute and chronic, *Acartia* acute, and *Skeletonema* 72-hour growth.

Quality assurance through BEQUALM is in place or currently running (JAMP, 1998; ICES, 2005). 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 (expected publication date: Autumn 2011).

Synergism between CEMP, MSFD and WFD

Though bioassays are not included as ecological quality elements in the monitoring for the Water Framework Directive (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 deN Heuvel *et al*., 2005; Maas *et al*., 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.

Thresholds and assessment tools

General

Thresholds for water bioassays are available. Effects measured include acute (e.g. mortality) or chronic endpoints (sub lethal endpoint such as growth, development and reproduction) and hence are generic indicators of toxicity of the water. Values of ECxx, LCxx, NOEC and LOEC 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.

Methods for water *in vivo* bioassays currently in JAMP

Water *in vivo* bioassays

The species recommended for water *in vivo* bioassays are:

• Copepod (*Tisbe battaglii* and *Acartia* sp); 48 hour exposure using mortality as the endpoint.

• Bivalves (*Crassostrea gigas*, *Mytilus* spp) embryos: 24 hour exposure using Percent Net Response as the endpoint.

• Sea urchin (*Paracentrotus lividus*): 24 hour embryo exposure using percent 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, a control and positive control are used. The control is a “pristine water” of known water quality and characteristic 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.

Assessing the data

The data for water bioassays can be considered in much the same way as for sediment bioassays and 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, 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 becomes available (see also Table 3 below). Responses greater than two times 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 two times background should be categorized as a cause for concern and prompting further sampling in terms 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 a ECx (percent dilution causing a x% reduction in the endpoint) or toxic units (TU=100/ECx) approach. A phased Toxicity Identification Evaluation (TIE) can be conducted to further describe the nature of the toxicity or potential toxicants present.

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 bataglii*, 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. From resulting samples, the averaged per lab/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 lab/countries datasets. The current assessment thresholds are given in Table 3.

Table 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	
lab	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
Stdv	Standard deviation of the individual exposures

Table 3. 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

Ecotoxicological assessment criteria for in vivo and in vitro bioassays

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

Assessment framework: metric and criteria

Experience in the Netherlands

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 selected: 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);

• 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 1.

Proposed metric and criteria for use of *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 however be noted regarding consistency with standards for individual substances:

a) Concerning the method: the same methods have been used for the metric as for substance standards, as described in the RIVM report ‘Guidance Document on Deriving Environmental Risk Limits (Traas, 2001):

i) 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 PAF5 (PAF = potentially affected fraction);

ii) 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, though the principle is the same.

b) As regards the factor for MPE/SE: a factor 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. In bioassays, where samples from the field are used, this effect has already been taken into account, and a factor 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 fresh water, 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 (so including SE).

Standard for *in vivo* bioassays for surface water

Method 1. Standard with 'preliminary effect assessment' (Cf = concentration factor compared with the untreated sample (original water sample); this can be seen as the 'assessment factor' applied in the case of three acute or chronic tests from different taxonomic groups).

Table 4. Details of the metrics for surface water.

Acute tests	
NE (negligible effect)	in 3 acute tests effect = 0 (in practice < EC50), Cf = 100
MPE (maximum permissible effect)	in 3 acute tests effect = 0 (in practice < EC50), Cf = 10
SE (serious effect)	in 1 acute test effect ≥ EC50, Cf = 10 or in 2 acute tests EC20< effect < EC50, 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 ≥ EC50, Cf = 1 or in 2 chronic tests NOEC < effect < EC50, Cf = 1
EC50 = Mean effective concentration, produces a 50% effect in the bioassay	
NOEC = no-observed-effect concentration	

Method 2. Standard with 'refined effect assessment' (PAF approach; see Figure 2)

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 (2002)).
- For chronic values a species sensitivity distribution is assessed following a log-logistic distribution (Traas (2000)).
- The extent to which the PAF5 (for the MPE) and PAF50 (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 (PAF5)) is defined and divided by 10. This gives the concentration factor 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 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. The choice of chronic or acute will depend partly on the specific circumstances at the locations studied: the compartment to be assessed, knowledge of the degree of pollution, etc. 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 EC50 values were extrapolated to chronic NOEC values with a factor 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 the MPE or SE was exceeded.

Another example of toxicity-based assessment is illustrated in Table 1. Water samples from the surface water monitoring programme of the Western Scheldt estuary (NL) in the period 2000–2005 were extracted using 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 5 shows the results of a preliminary effect assessment using the test results of the three bioassays.

Table 5. Indication of toxicity in surface water of the Western Scheldt estuary on basis of three different bioassay responses allowing a preliminary effect assessment as proposed in Maas *et al*., 2003.

		Cf (ECf50)*			**Cf (MTE)**
location	**date**	Daphnia	Algae	Microtox	(from PAF5)
SvOD-1	12-2-00	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)

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 Percent Net Response (PNR); values range from 0 to 100, where 100 indicates that no oyster embryos developed. A value of 20 or more PNR is regarded as an adverse but negligible effect, a value of between 50 and 80 cause for concern (maximum permissible effect) and in excess of 80 a serious effect. PNR values of between 20–50 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 *et al*., 1996). More recently SPMD extraction procedures have been used successfully (Thomas *et al*., 1999; 2000) and we have developed a battery of bioassay tools to use which include; 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 Ahr receptor-based assay. The data has 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 EC50) are generally;

- >1000 at distant offshore station such as the ICES Reference Stations;
- 500–1000 offshore stations such as the western English Channel;
- 200–500 intermediate stations;
- 50–200 inshore stations;
- 10–50 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 that this has to be interpreted within the limitations and restrictions of the chemical process (see 5.3 above).

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 of 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 and in particular 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.

References

Åkerman, J. and Smit. T., 2003. Effect directed analyses in surface water of the Wadden Sea and Eems-Dollard (in Dutch. RIKZ working document RIKZ/AB/2003.607X.

Åkerman, J. *et al*., 2004. Stoffen in de Noordzee in de Nederlandse kustzone in 2003. Internal report nr.2004.040. RWS-RIKZ, The Hague, The Netherlands.

Aldenberg, T. and Jaworska J., 2000. Uncertainty of the hazardous concentration and fraction affected for normal species sensitivity distributions. Ecotox. Environ. Saf. 25, 48–63.

CIS, 2003. Guidance on Monitoring for the Water Framework Directive. WFD Common Implementation Strategy Working Group 2.7 Monitoring. 23 January 2003.

Den Besten, P.J. and Munawar, M. (eds). 2005. Ecotoxicological testing of marine and freshwater ecosystems: emerging techniques, trends and strategies. CRC Press. Boca Raton, FL USA: 271 pp.

Derksen, J.G.M., Spier, J.L., Maas, H. and Vethaak, A.D., 2004. Bioassays onder de loep. Selectie van bioassays voor zoet, zout en brak oppervlaktewater. RIZA 2004.012; RIKZ 2004 029; ISBN 9036956366.

Hartwell, S.I. 1998. Demonstration of a toxicological risk ranking method to correlate measures of ambient toxicity and fish community diversity. Environmental Toxicology and Chemistry 16: 361–371.

Hendriks, A.J., Maas-Diepeveen, J.L., Noordsij A. and van der Gaag., M.A., 1994. Monitoring response of XAD concentrated water in the Rhine Delta: A major part of the toxic compounds remains unidentified. Water Res. 28: 581–598.

Hill, I.R, Matthiessen P. and Heimbach F., 1993. Guidance document on sediment toxicity tests and bioassays for freshwater and marine environments. Workshop on sediment toxicity assessment held at Slot Moermond Congress Centre, Renesse, The Netherlands, 8–10 November 1993. SETAC publication.

Hylland, K., Becker, G., Klungsøyr, J., Lang, T., McIntosh, A., Serigstad, B., Thain, T., Thomas, K., Utvik, T.I.R., Vethaak, D., Wosniok, W., 2002. An ICES workshop on biological effects in pelagic ecosystems (BECPELAG): summary of results and recommendations. ICES ASC 2002 CM 2002/X:13.

Hylland, Ketil *et al*. (eds). 2006. Biological effects of contaminants in marine pelagic ecosystems. SETAC Special Book Publications (in press).

ICES, 2004. Report of the Working Group on biological effects of contaminants. Report 2004. ICES Marine Habitat Committee ICES CM 2004/E:04 Ref. ACME.

ICES, 2005. Report of the Working Group on biological effects of contaminants. Report 2005. ICES, CM/2005./E:08. Ref. ACME.

JAMP (Joint Assessment and Monitoring Programme), 1998. JAMP guidelines for general biological effects monitoring. Oslo and Paris Commissions, 15 p.

Karbe, L., 1992. Toxicity of surface microlayer, subsurface water and sediment-elutriates from the German Bight: summary and conclusions. Marine Ecology Progress Series 91:197–201.

Kirby, M.F., Blackburn, M. A., Thain, J.E. and Waldock. M.J., 1998. Assessment of water quality in estuarine and coastal waters of England and Wales using a contaminant concentration technique. Marine Pollution Bulletin 36: 631–642.

Maas, J.L., van de Plassche, E.J., Straetman, A., Vethaak, A.D. and Belfroid, A., 2003. Normstelling voor bioassays. Uitwerking voor oppervlaktewater en waterbodems. RWS-RIZA/RIKZ report 2003.007.

Maas, J.L., van de Heuvel-Greve, M., 2005. Opportunities for bio-analysis in WFD chemical monitoring using bioassays. RWS RIZA/RIKZ. Internal report.

Matthiessen, P., Thain, J.E., Law, R.J. and Fileman, T.W. 1993. Attempts to assess the environmental hazard posed by complex mixtures of organic chemicals in UK estuaries. Marine Pollution Bulletin 26: 90–95.

Oris, J. and Klaine, S., 2000. Whole effluent toxicity testing. Environmental Toxicology and Chemistry 1–256.

Péry, A., Flammarion, P., Vollat, B., Bedaux, J.J.M., Kooijman, S.A.L.M. and Garric, J., 2002. Using a biologic-based model (DEBTOX) to analyze bioassay in ecotoxicology. Opportunities and recommendations. Environmental Toxicology and Chemistry 21: 459–465.

Peters, C., Becker, S., Noack, U., Pfitzner, S., Bülow, W., Barz, K., Ahlf, W. and Berghahn, R., 2002. A Marine Bioassay Test Set to Assess Marine Water and Sediment Quality – its Need, the Approach and First Results. Ecotoxicology 11 (5): 379–383.

Power, E.A. 2004. International Trends in Bioassay Use for Effluent Management. Ecotoxicology 13 (5): 377–398.

Stebbing, A.R.D., Dethlefsen, V., Carr. M.,1992. Results of the Bremerhaven workshop on biological effects of contaminants. Mar. Ecol. Prog. Ser., 91, 361 pp.

Thain, J.E. 1991. Biological effects of contaminants: Oyster (*Crassostrea gigas*) embryo bioassay. Techniques in Marine Environmental Sciences, No. 11. 12 pp.

Thain, J.E. and Kirby, M. 1996. Improving the sensitivity of biological water quality measurements in marine waters. In Proceedings of the Scientific Symposium on the North Sea Quality Status Report 1993. 18–21 April 1994, Ebeltoft, Denmark, pp 151–156. Danish Environmental Protection Agency.

Thomas, K.V., Hurst, M.R., Matthiessen, P., McHugh, M., Smith, A., Waldock, M.J. 2002. An assessment of *in vitro* androgenic activity and the identification of environmental androgens in United Kingdom estuaries. Environmental Toxicology and Chemistry 7: 1456–1461.

Thomas, K.V., Benstead, R.E., Thain, J.E. and Waldock, M.J. 1999. Toxicity Characterization of Organic Contaminants in Industrialized UK Estuaries and Coastal Waters. Marine Pollution Bulletin38: 925–932.

Thomas, K.V., Hurst, M.R., Lavender, J., Matthiessen, P., Thain, J.E., Waldock, M.J., 2000. Characterising hazardous substances in the UK marine environment. ICES ASC CM 2000/S:11.

Tonkes, M., den besten, P.J. and Leverett, D. 2005. Bioassays and tiered approaches for monitoring surface quality and effluents. In: Den Besten, PJ and M Munawar (eds). Ecotoxicological testing of marine and fresh-water ecosystems: emerging techniques, trends and strategies. CRC Press. Boca Raton, FL USA: 43–86.

Traas, T.P. (ed.). 2001. Guidance Document on deriving Environmental Risk Limits. RIVM rapport nr. 601501 012.

US EPA, 1991. Methods for Aquatic Toxicity Identification Evaluation: Phase I Toxicity Characterisation Procedures. EPA Report EPA-600/6-91/003. US EPA, Duluth, MN, USA.

US EPA, 1993. Methods for Aquatic Toxicity Identification Evaluation: Phase II Toxicity Characterisation Procedures. EPA Report EPA-600/R-92/08. US EPA, Duluth, MN, USA.

US EPA, 1995. Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to West Coast Marine and Estuarine Organisms. National Exposure Research Laboratory, Office of Research and Development. United States Environmental Protection Agency. Cincinnati. 648 pp.

van den Heuvel-Greve, Maas, J.L. and Vethaak, A.D.. 2005. Ecoassays: an exploratory study on the possible application of ecoassays in the context of the Water Framework Directive. RWS-RIKZ/RIZA. Report nr RIKZ/2005.019.

Zwart, D. de and Sterkenburg, A., 2002. Toxicity-based assessment of water-quality. In: Posthuma, L., G.W. Suter II and T.P. Traas (Eds). Species Sensitivity Distributions in Ecotoxicology. Lewis Publishers.

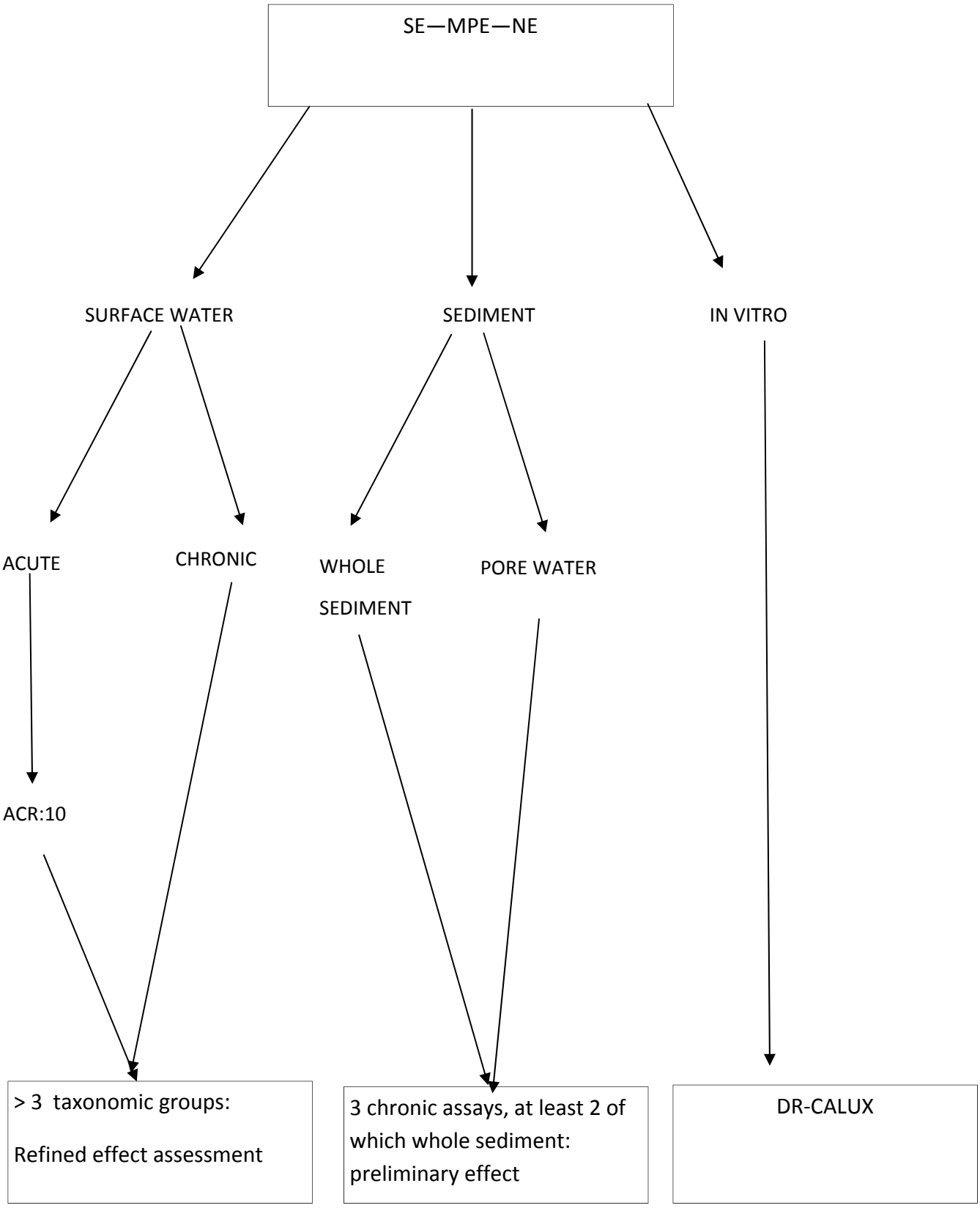


Figure 1. Summary of the metrics based on *in vivo* bioassays for surface water (and sediment, and on in vitro bioassays) (ACR: acute-chronic ratio; PAF: potentially affected fraction).

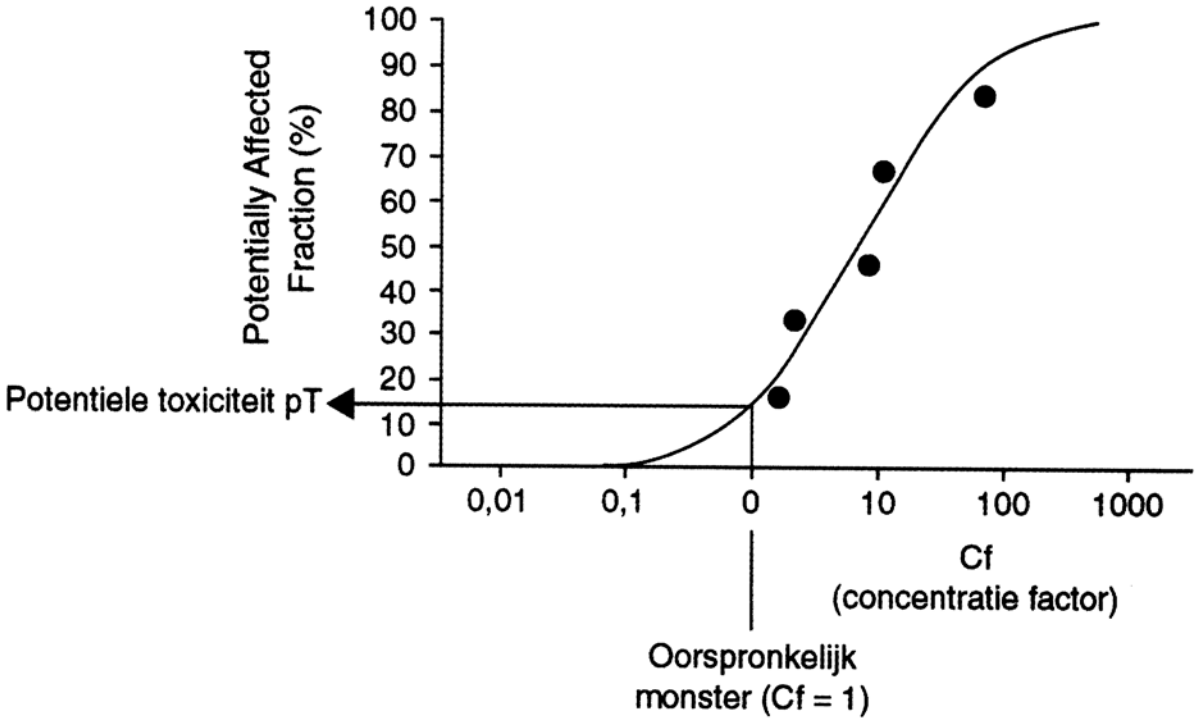


Figure 2. Use of a response curve to estimate the potentially affected fraction (PAF%). (Oorspronkelijk monster = original sample)

Technical Annex on recommended packages of chemical and biological methods for monitoring on a determinant basis

Review of Co-ordinated Environmental Monitoring Programme (CEMP) requirements

This agenda item was addressed by reviewing the chemical determinants listed in the OSPAR 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:

a) 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;

b) 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 allows for temporally integrated sampling of contaminants in water. (Guidelines for the application of passive samplers are available from ICES WGMS);

c) The biological effects techniques applied to these packages of methods are listed either in the ICES WGBEC recommended techniques list (WGBEC, 2007) or form part of the fish and shellfish methods packages proposed in the draft 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 for monitoring selected fish species, shellfish (mussels) and bioassays (sediment, water and *in vitro* tests);

d) 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;

e) These packages of methods should be considered supplemental to the existing JAMP guidelines for contaminant specific (OSPAR Agreement 2003-10) and general (OSPAR Agreement1997-7) biological effects monitoring as well as the JAMP Guidelines on contaminants in biota (OSPAR Agreement 1999-2) and sediment (OSPAR Agreement 2002-16). The JAMP guidelines provide more detailed background on the biological effects and chemical analysis methods referred to here and the necessary co-factors 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.

Technical Annex on recommended packages of chemical and biological methods for monitoring on a determinant basis

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 for example 100 times higher than the Background Assessment Concentration (BAC) (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);
- PFOS in sediment, biota and water (Appendix 12);
- Polychlorinated dibenzodioxins and furans in biota and sediment (Appendix 13).

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 film (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 and it is recommended that it is applied only in areas where lead contamination is perceived to be a problem or where chemical monitoring indicates that concentrations are significantly above background, for example.

ALA-D is relevant for fish only. 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 due to the lack of standardised methods, Quality Assurance (QA) 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 1 below. *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.

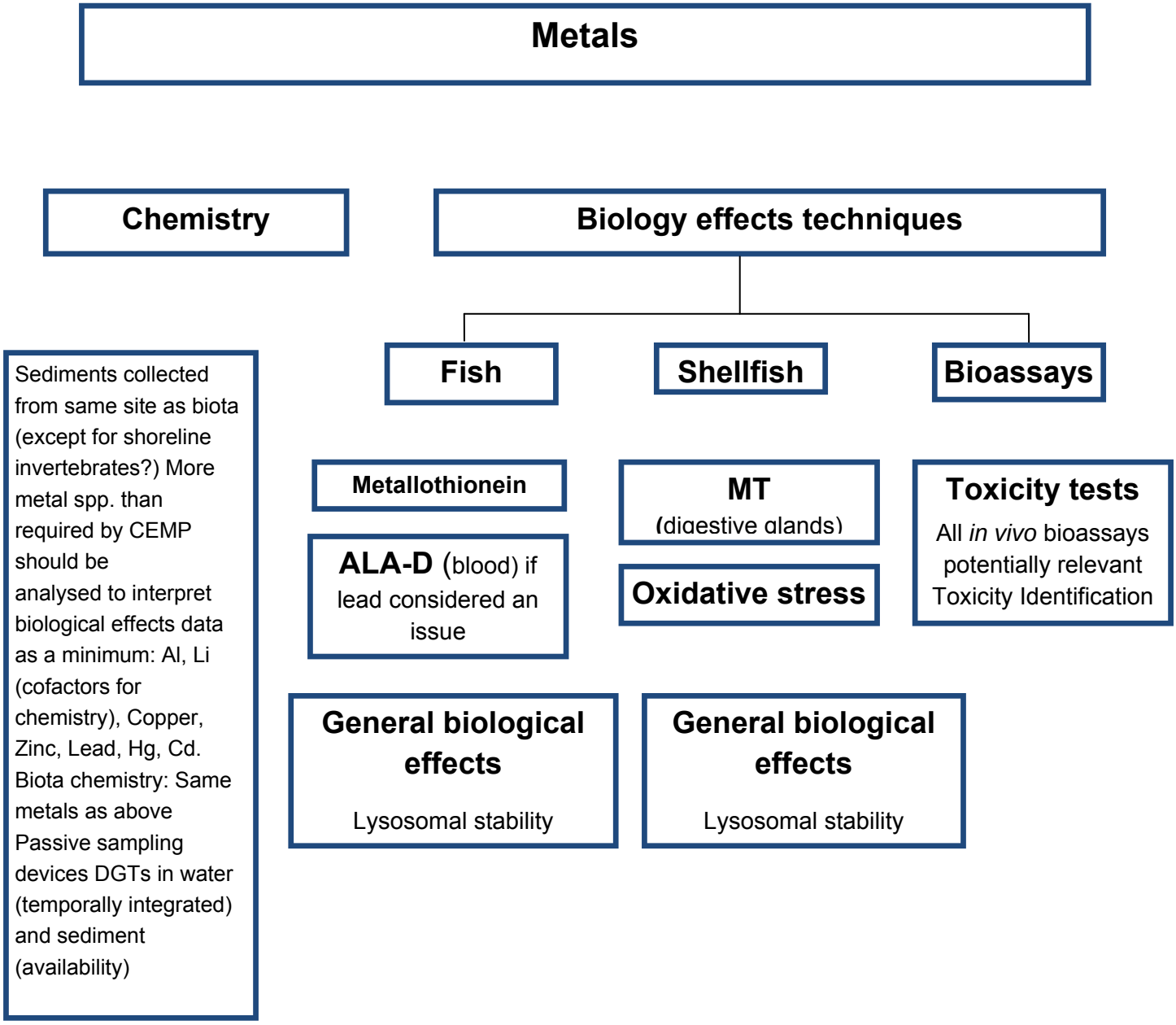


Figure 1. Package of chemical and biological effects methods relevant to monitoring for metals. The most specific / relevant biological effects methods are highlighted (bold, shade).

Methods package for PCBs, polychlorinated dibenzodioxins and furans

Due to the similarity of their toxicological effects, a single methods package was proposed for both PCBs and polychlorinated dibenzodioxins and furans. In addition to the OSPAR CEMP required determinants, additional CBs may cause biological effects and their analysis should be included in an integrated monitoring approach. These include co-planar 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 given below in Figure 2. DR-Calux is considered the most useful in vitro bioassay technique although chronic in vivo bioassays may also be relevant.

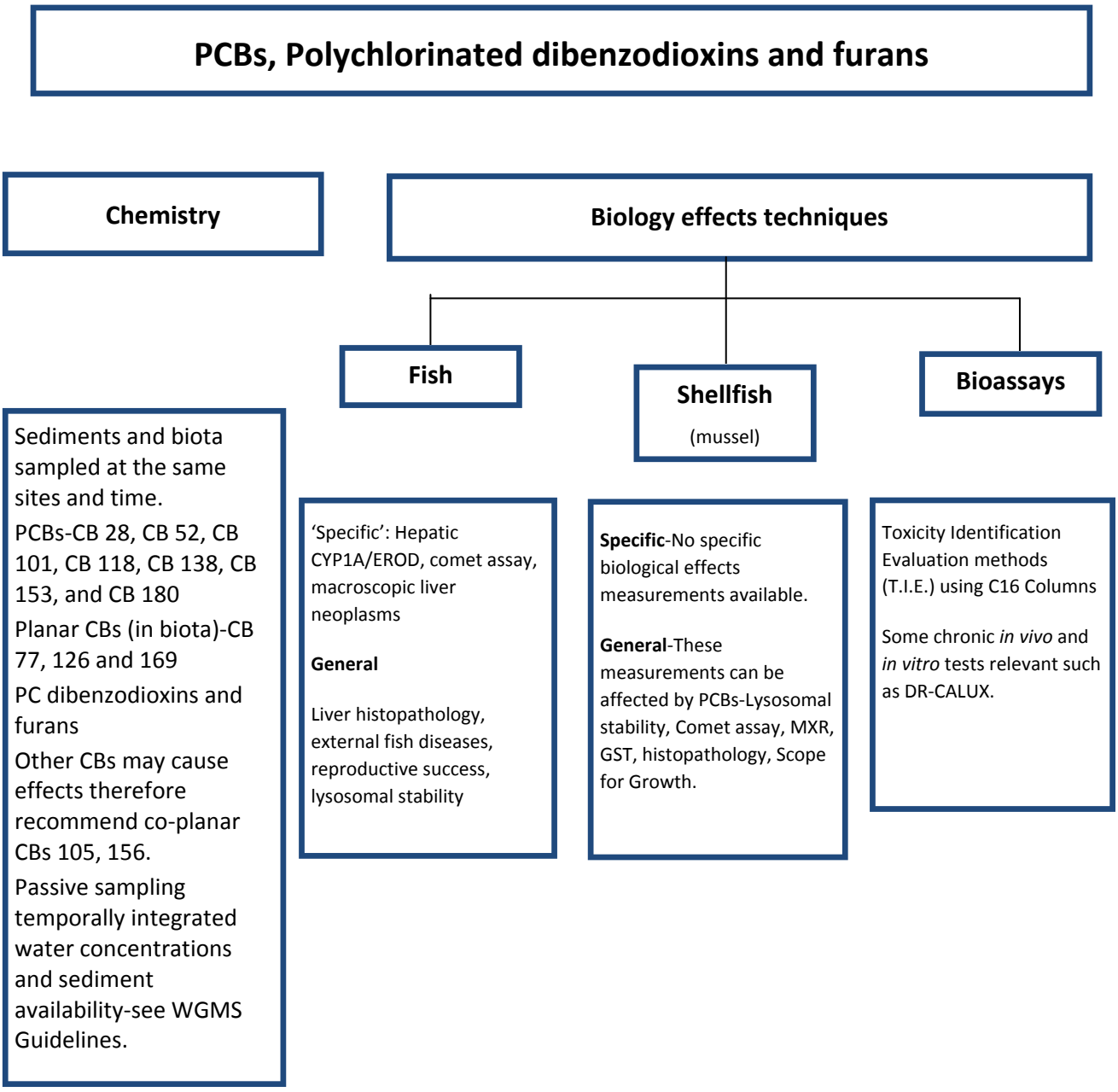


Figure 2. Package of chemical and biological effects methods relevant to monitoring for PCBs polychlorinated dibenzodioxins and furans. The most specific/relevant biological effects methods are highlighted (bold, shade).

Methods package for PAH and alkylated PAH

Due to similar toxicological effects, a single package of methods is proposed for PAH and alkylated PAH (Figure 3). The package of methods is similar to Figure 2 above although chemical determinants should be analysed in sediment and shellfish for biota only. Due to rapid metabolism in finfish, PAH should be analysed

as metabolites in bile rather than parent compounds in liver or flesh. As above, passive sampling should also be applied where possible.

Additional specific biological effects are applicable for PAH/alkylated PAH. 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 3 below.

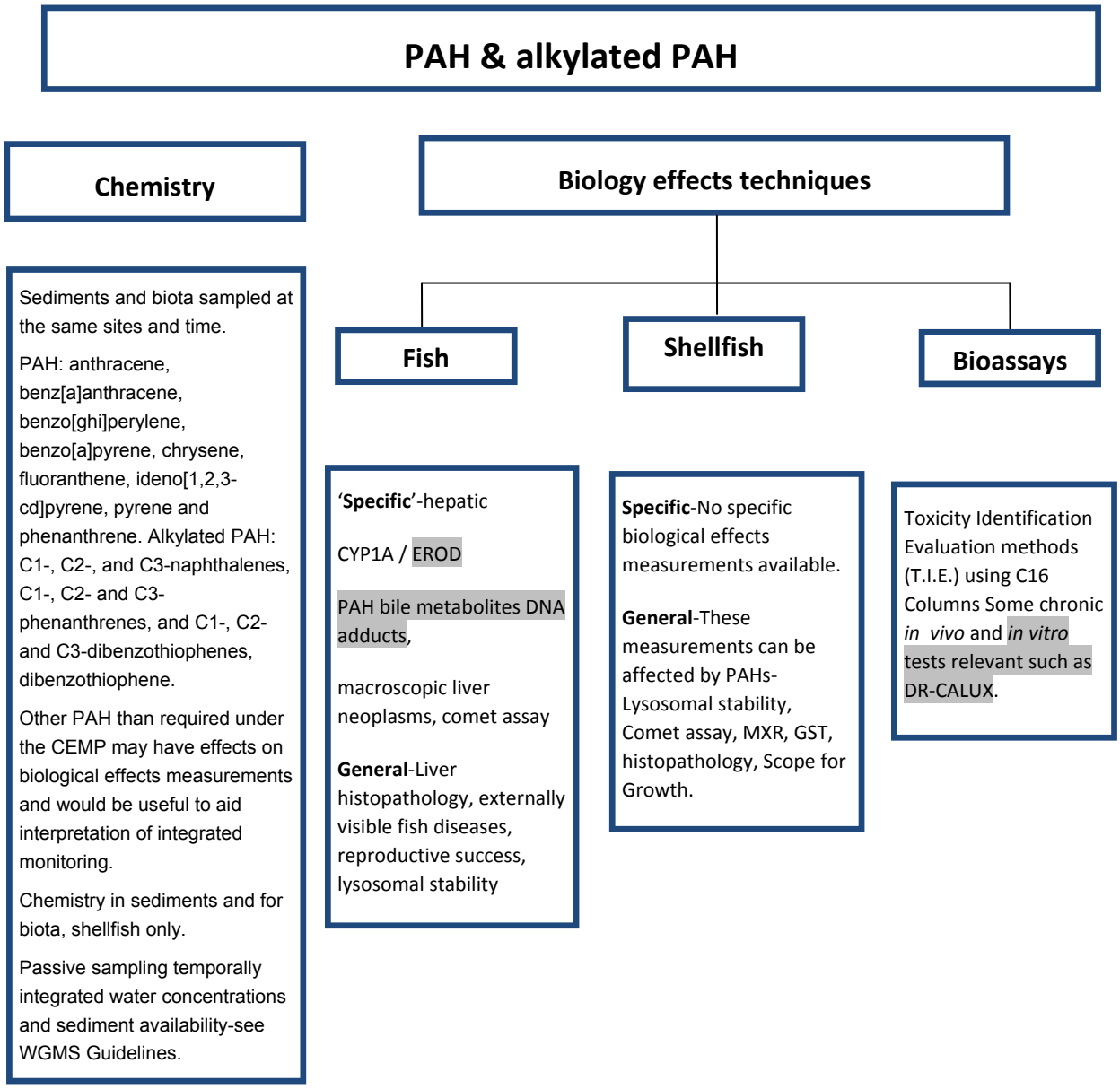


Figure 3. Package of chemical and biological effects methods relevant to monitoring for PAH and alkylated PAH. The most specific / relevant biological effects methods are highlighted (bold, shade).

Organotins

It was felt that the package of methods appropriate for 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 the future. It was also noted that bivalve embryo bioassays are sensitive to dissolved TBT at ng/L level.

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 these compounds. 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.

PFOS

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 ED-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 bio-analysis 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.

References

ASMO 2007a. Proposal for revising the OSPAR Coordinated Environmental Monitoring Programme (CEMP). ASMO 07/13/1.

ASMO 2007b. Draft JAMP guidelines for integrated monitoring and assessment of contaminants and their effects. ASMO 07/6/8-E.

WGBEC 2007. Report of the ICES Working Group on Biological Effects of Contaminants, 2007.

Technical Annex on sampling and analysis for integrated chemical and biological effects monitoring in fish and shellfish

Introduction

ICES/OSPAR 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 Strategy for Hazardous Substances. 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?

Integration of chemical and biological effects measurements in OSPAR CEMP

The primary means of addressing these questions on an OSPAR wide basis is the Coordinated Environmental Monitoring Programme (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 of OSPAR request 2008/8.

The integrated approach described in the Guidelines is been based around 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 reproduced below as Figures 1 and 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 due to the possible identification of the substances contributing to the observed effects.

The structure of each of the schemes recognises 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 recognised 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. While 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, particularly if chemical analysis at concentrations known to be associated with the effects is difficult. However, generally such close relationships are rare. For example, a range of effects measurements have been applied to the effects of planar organic contaminants in the sea, i.e.

- the concentration of PAH-metabolites in fish bile;
- CYP1A/EROD induction;
- Indices of genotoxicity (e.g. DNA adducts of PAH, COMET assay, micronucleus assay, etc.);
- liver (microscopic) neoplasms;
- liver histopathology.

However, these effects show varying degrees of specificity for PAH as opposed to other planar organic contaminants such as planar CBs, 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 while subcellular responses can commonly be linked to a substances that have the potential to 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 1 and 2 under the headings of sub-cellular responses, tissues responses and whole organism responses. Sub-cellular responses such as EROD, bile metabolite concentrations and metallothionein are recognised as biomarkers of exposure to contaminants, while whole organism and tissue level responses are more clearly markers of effect.

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:

1. the sampling and analyses of same tissues and individuals;
2. the 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;
3. the sampling of water, the water column and sediments at the same time and location as collecting biota; and
4. the 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 1 and 2. The numbers of individual organisms required are driven primarily by the assessment of external diseases and macroscopic liver nodules (fish) and histopathology (bivalves), since these require the largest number of individuals. A sub-sample of individuals within the primary sample is further sampled for liver histopathology (fish) and biomarkers (fish and bivalves) to meet Requirements 1 and 2 above.

In the specified target species, further sub-sampling of the same individuals for chemical analysis is often restricted by insufficient remaining tissue, e.g. liver in fish. In order to meet Requirement 2, sub-samples 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.

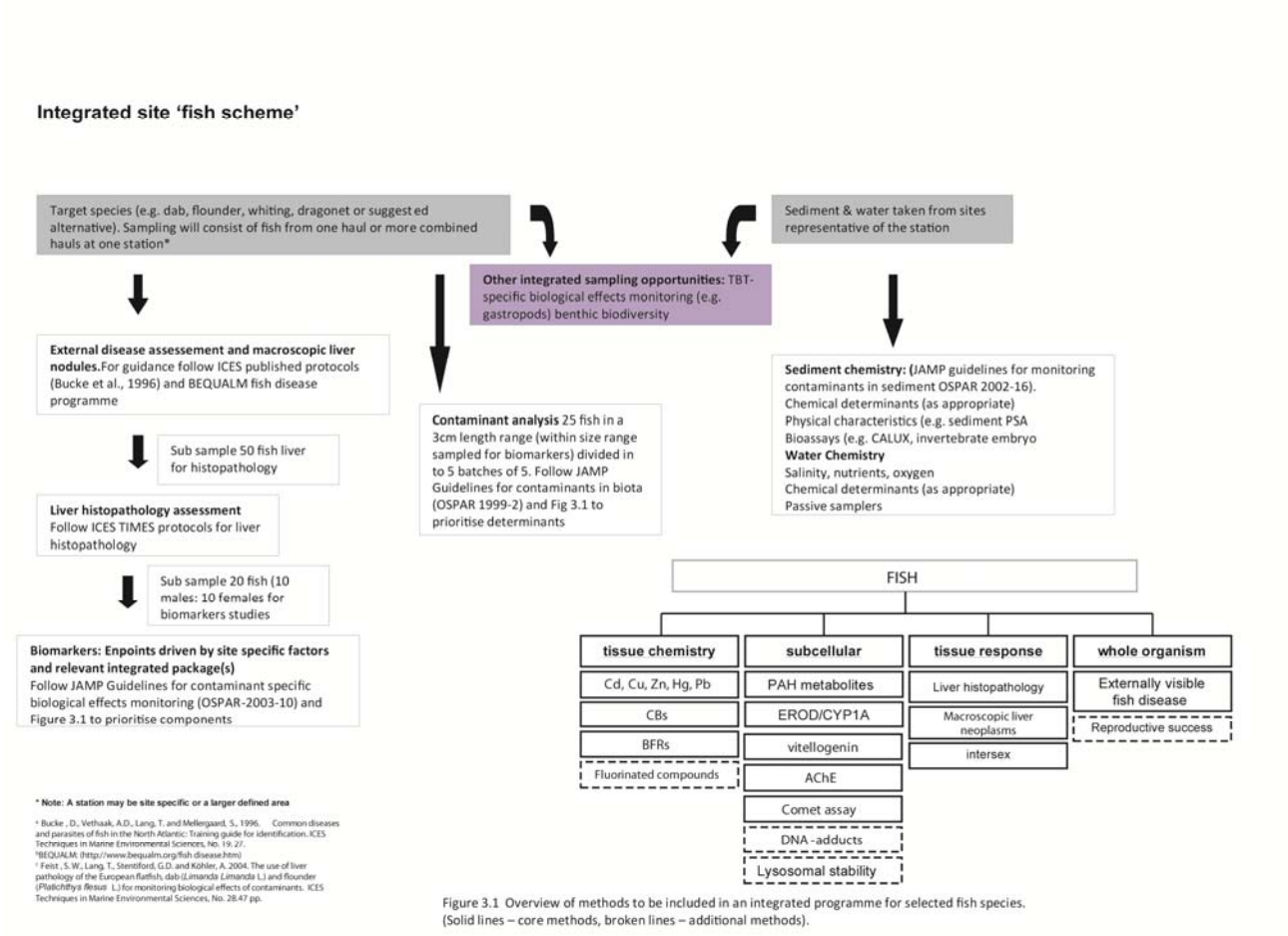


Figure 1. Sampling strategy for integrated fish monitoring

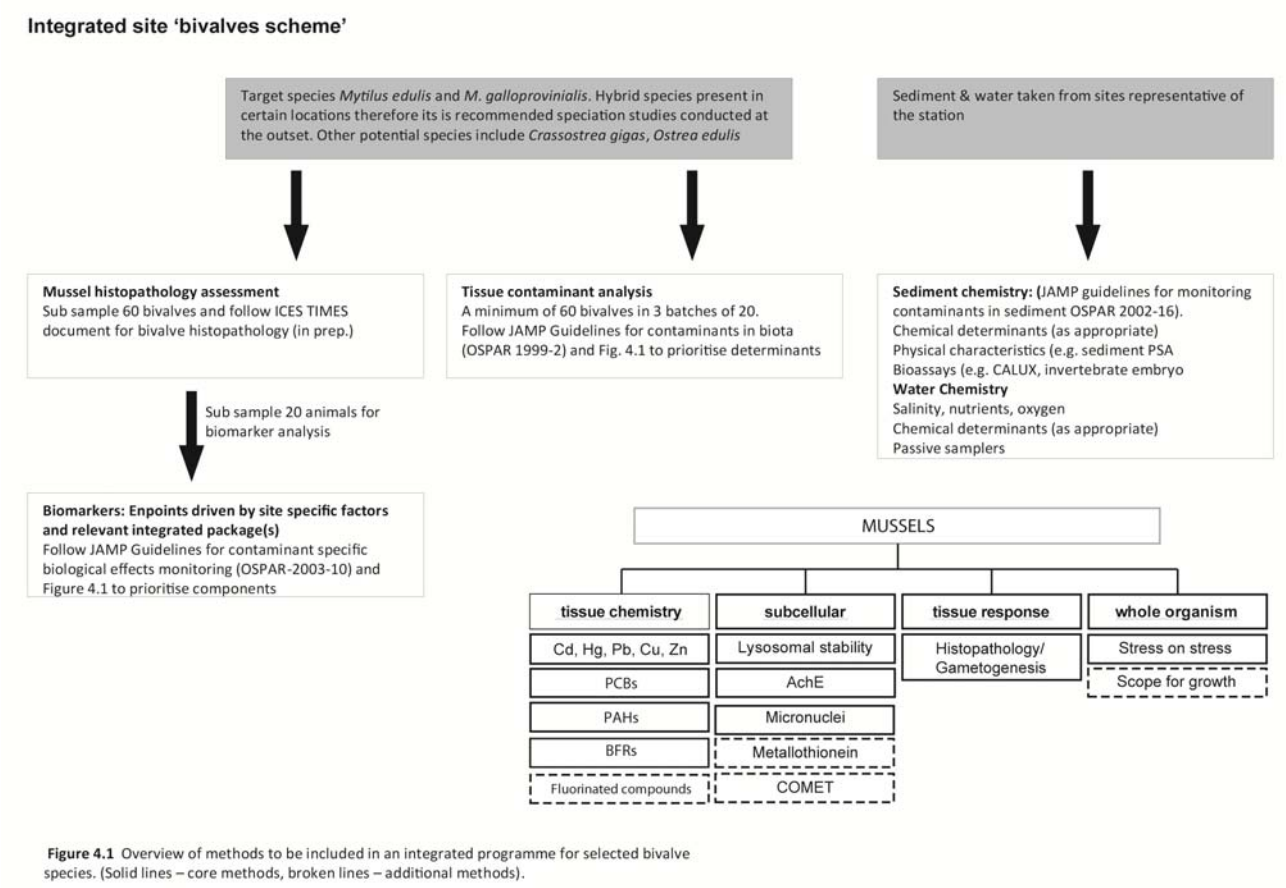


Figure 2. Sampling strategy for integrated bivalve monitoring.

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 1–3 cover methods to be used for integrated fish, bivalve and gastropod monitoring, Tables 4 and 5 cover methods for monitoring of water and sediments.

Table 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 fulfill 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 ij the survey area, and meet the purposes of the monitoring programme.

SUBJECT	PARAMETER	COMMENT
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 (eg., using UV-fluorescence spectrometry) may be employed under specific circumstances (e.g. after oil spill or PAH-related point source discharges). Besides 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 1 and 2 above	Additional opportunities for the inclusion of new methods is 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 harmonised between monitoring authorities. An appropriate value would be 30 minutes, but may be less than this if conditions require.	The purpose is to standardise 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 hours.	Storage for longer periods or under poor conditions can stress the fish and alter some biomarker responses.

Table 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 between 40–55mm. 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 has still 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 ij 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 (eg., using UV-fluorescence spectrometry) may be employed under specific circumstances (e.g. after oil spill or PAH-related point source discharges). Besides 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.

SUBJECT	PARAMETER	COMMENT
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 1 and 2 above	Additional opportunities for the inclusion of new methods is likely to emerge through the implementation of MSFD and as science develops. Potential examples include indicators of immuno-competence, 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 normalisation.
Sampling depth	Subtidal or intertidal mussels can be used. Deployed mussels offshore can be positioned at depths 0–8m	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 hours. They should be transported in an insulated container at 4oC in a damp atmosphere maintained by absorbent materials (such as seaweed and/or paper towel) wetted with seawater.	

Table 3. Overview of methods and species for integrated gastropod/organotin monitoring (2007 WKIMON Report, revised).

SUBJECT	PARAMETER	COMMENT
Species	Intertidal species: *Nucella lapillus* *Nassarius reticulata* *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 Guideline) ICES TIMES document on intersex in *Littorina* provides methodological advice.	
Supporting parameters	Shell length, organotin compounds in sediment.	

Table 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 in the case of specific events, such as blooms affecting fish health

Table 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 normalisation 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.

Technical Annex for Integrated chemical and biological monitoring of Mussel (Mytilus sp.)

Background

The basis for the technical annex is the mussel integrated monitoring strategy incorporating biological effect techniques at the sub-cellular, tissue and whole organism responses and tissue chemistry. This is outlined below (Figure 1):

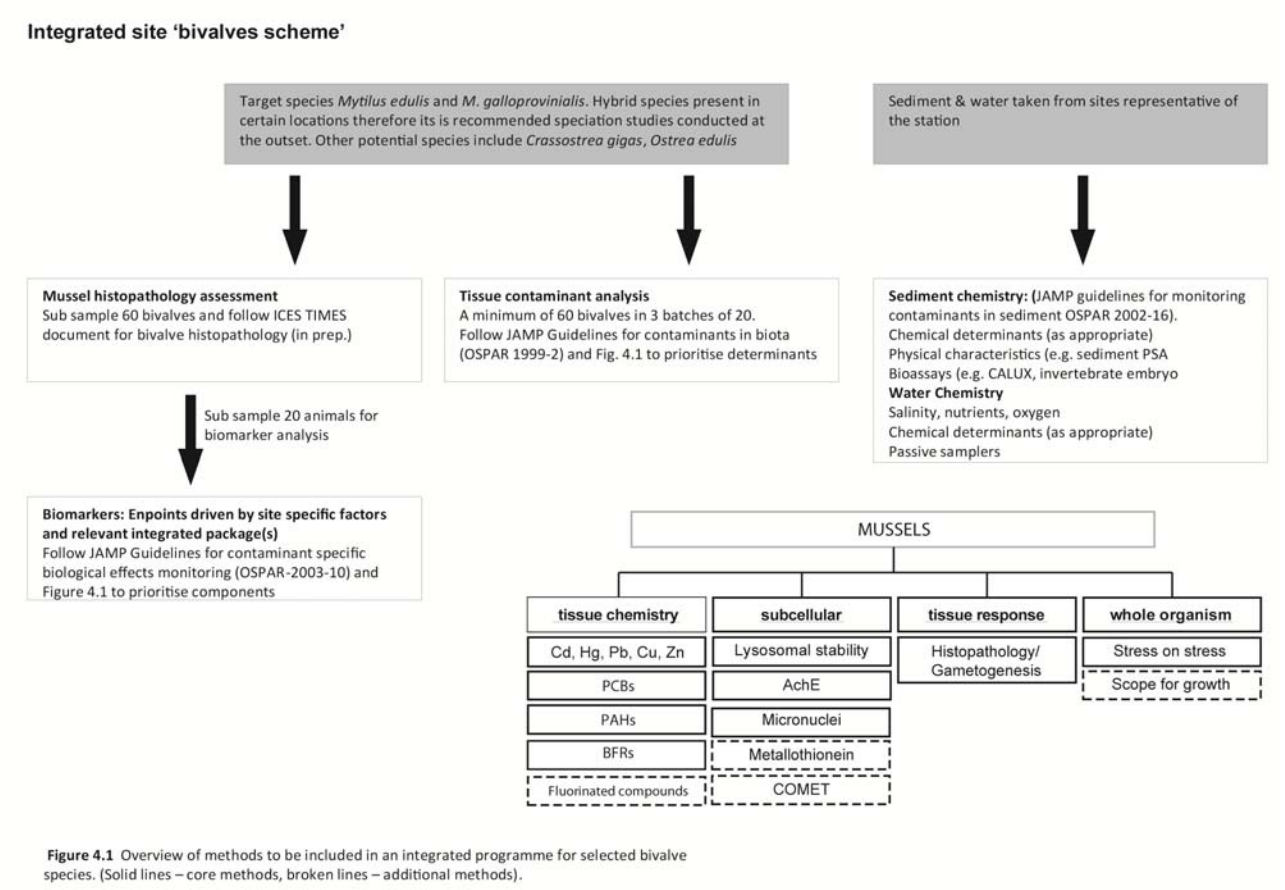


Figure 1. Sampling strategy for integrated bivalve monitoring.

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

Purpose of work

The integrated approach described above can be used for:

- *Status and trend monitoring*; contaminant and biological effect responses are measured over geographic areas and repeated over time. The purpose here may be to compare biological effect responses between sites, to compare changes in response with time and to observe if the "health status" is improving, at a steady state or declining.
- *Investigative monitoring*; 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*; usually in relation to risk assessment at pollution sites e.g. oil platform investigations.

Offshore and coastal

Mussels (*Mytilus* species) are infrequently found in the sub littoral zone. But populations do exist in shallow waters 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; 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); standardisation of origin (same source/supply), size and species. Disadvantages are: cost of deployment in respect of mooring systems and ship time for deployment and retrieval; in addition some techniques require immediate sampling and analysis which may not be feasible on a research vessel offshore.

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

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 then organisms can be caged on the seashore or in estuaries on piers or similar structures.

Sampling information

Details 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 Lat. Long.

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 and information on currents and stratification if available, water temperature 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, ideally the mussels must be sampled in a uniform manner between sites i.e. tidal height and similar salinity profile.

Confounding factors

For *in situ* transplants/caging the mussels must be deployed for at least three weeks 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, of particular importance for caging work in estuaries.

Temperature: mussels on the shoreline can be subject to extremes of temperature, cold in the winter and extreme heat in the 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 and late summer and autumn intense 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 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 then 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 account of in the experimental design.

Supporting measurements

Condition index; dry meat relative to whole live weight or internal shell volume.

Gonad state; index of reproductive state.

Lipid content; usually a 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, DO, 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. Prioritised 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 hours to eliminate gut contents (e.g. sediment, etc.). 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.

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 the table below (Table 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 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 hr	Ca 10 hr	Crucial
AChE (10)	Immediate in field	Not applicable	Stored immediately in liquid nitrogen
Mt (10)	Any time within 24 hr on live mussel	Not applicable	Take tissue sample – freeze in liquid nitrogen
COMET	Within 24 hr	Store for no more than 24 hr in cool damp conditions. Must be consistant in startegy	Do as quickly as possible
Micronuclei (20)	Within 3 days	None	Mussels can be kept out of water but cool
NRR (10)	Within 24 hr	Store for no more than 24 hr in cool damp conditions. Must be consistant in startegy	Do as quickly as possible
Lysosomal histochemical method (10)	Freeze immediately	Not applicable	In liquid nitrogen
Stress on stress (40)	Not aplicable	Transport at low temperatures for no more than 24 hr	Analysis done at 18°C
Histopathology and gametogenesis (30–50)	Sample immediately if possible	Anything more than 6 hr delay in sampling place in water for 48 hr acclimation	Dessication must be avoided, correct dissection to incude all organs
Tissue chemistry (50)	Place in 24 hr clean running sea water	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 and this is described below:

The technical procedure essential for correct mussel sampling for histology (taken from draft TIMES doc. under preparation, provided by J Bignell UK, Cefas):

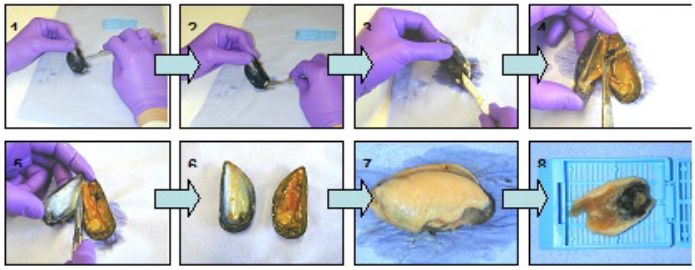
Insert scalpel into ventral byssal cavity and move knife down so it cuts the posterior adductor muscle.

Open shell and remove byssal thread.

Remove mussel from one shell half. Repeat for remaining half.

Analyse tissue for presence of parasites, pearls or other abnormalities.

Obtain a standardised section as shown in photographs 1–8 in order to include all organs of interest in one section and place into histo-cassette.



Samples should be preserved for a minimum of 24 hours in Bakers Formal Calcium, and subsequently transferred to 70% alcohol until processed.

The correct ratio of mussels to fixative is 30 samples per 800 ml (approx.) of fixative. This is the recommended volume of fixative to ensure adequate fixation.

Samples should be agitated periodically to ensure thorough fixation. A rocker plate facilitates this perfectly.

Methods to be used

These are listed in the mussel integrated strategy above. An overview of the methods is given in the table below (Table 2) with references to the analytical procedures.

Table 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 sub-lethal 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

Quality assurance

Wherever possible all analytical methods must be supported with quality assurance 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 MEDPOL, for internal QA a dual assessment with a colleague on the same samples is recommended.

Ache – not yet developed but include internal standard.

Mt – MEDPOL have intercalibration exercises, elsewhere there have been *ad hoc* intercalibrations and additionally an internal standard should be included.

SFG – none at present.

Stress on Stress – none at present but will be addressed by MEDPOL/ICES workshop in 2010.

Histology and gametogenesis – TIMES doc and circulation of reference material.

Lysosomal histochemical procedures – none currently available but include an internal standard.

Micronuclei formation – currently being addressed through MEDPOL and may be extended to include a wider participation.

COMET – none at present but being addressed through ICES WGBEC.

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 then the data should be identified in the reporting and data assessment.

Contaminants: reported in line with standard analytical procedures.

Supporting parameters

Essential; date and time of sampling, Lat. Long. position, organism length, whole weight, site characterisation (e.g. position on shore, or caging, DO, salinity, etc.); for caged studies the source of organisms and duration of exposure.

Desirable; identification of species particularly if in a hybrid zone.

References

Al-Sabti K and Metcalfe C. 1995. Fish micronuclei for assessing genotoxicity in water. Mutat Res 343:121–135.

Bocquené, G., Bellanger, C, Cadiou, Y., and Galgani, F. 1995. Joint action of combinations of pollutants on the acetylcholinesterase activity of several marine species. Ecotoxicology, 4: 266–279

Cajaraville, M.P., Díez, G., Marigómez, J.A., and Angulo, E. 1990. Responses of the basophilic cells of the digestive gland of mussels to petroleum hydrocarbon exposure. Diseases of Aquatic Organisms, 9: 221–228.

Cajaraville, M.P., Marigómez, J.A., Díez, G., and Angulo, E. 1992. Comparative effects of the WAF of three oils on mussels. 2. Quantitative alterations in the structure of the digestive tubules. Comparative Biochemistry and Physiology, 102C(1): 113–123.

Cajaraville, M.P., Abascal, I., Etxeberria, M., and Marigomez, I. 1995. Lysosomes as cellular markers of environmental pollution time-dependent and dose-dependent responses of the digestive lysosomal system of mussels after petroleum hydrocarbon exposure. Environmental Toxicology and Water Quality, 10: 1–8.

Cheung, V.V., Wedderburn, R.J., and Depledge, M.H. 1998. Molluscan lysosomal responses as a diagnostic tool for detection of a pollution gradient in Tolo Harbour, Hong Kong. Marine Environmental Research, 46: 237–241.

Etxeberria, M., Cajaraville, M.P., and Marigomez, I. 1995. Changes in digestive cell lysosomal structure in mussels as biomarkers of environmental stress in the Urdaibai Estuary (Biscay Coast, Iberian Peninsula). Marine Pollution Bulletin, 30: 599–603.

Fernley, P.W., Moore, M.N., Lowe, D.M., Donkin, P., and Evans, S. 2000. Impact of the *Sea Empress* oil spill on lysosomal stability in mussel blood cells. Marine Environmental Research, 50: 451–455.

Grisolia CK and Cordeiro CMT. 2000. Variability in micronucleus induction with different mutagens applied to several species of fish. Genetics and Molecular Biology 23(1):235–239.

Lin, S.Y., and Steichen, D.J. 1994. A method for determining the stability of lysosomal membranes in the digestive cells *of Mytilus edulis.* Marine Ecology Progress Series, 115: 237–241.

Lowe, D.M., and Fossato, V.U. 2000. The influence of environmental contaminants on lysosomal activity in the digestive cells of mussels *(Mytilus galloprovincialis)* from the Venice Lagoon. Aquatic Toxicology, 48: 75–85.

Lowe, D.M., Soverchia, C, and Moore, M.N. 1995. Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquatic Toxicology, 33: 105–112.

Marigomez, I., and Baybay-Villacorta, L. 2003. Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. Aquatic Toxicology, 64: 235–257.

Regoli, F. 1992. Lysosomal responses as a sensitive stress index in biomonitoring heavy-metal pollution. Marine Ecology Progress Series, 84: 63–69.

Rickwood, C.J., and Galloway, T.S. 2004. Acetylcholinesterase inhibition as a biomarker of adverse effect. A study of *Mytilus edulis* exposed to the priority pollutant chlorfenvinphos. Aquatic Toxicology, 67: 45–56.

Ringwood, A.H., Conners, D.E., and Di Novo, A. 1998. The effects of copper exposures on cellular responses in oysters. Marine Environmental Research, 46: 591–595.

Ringwood, A.H., Connors, D.E., and Hoguet, J. 1998. Effects of natural and anthropogenic stressors on lysosomal destabilisation in oysters, *Crassostrea virginica.* Marine Ecology Progress Series, 166: 163–171.

Ringwood, A.H., Conners, D.E., and Keppler, C.J. 1999. Cellular responses of oysters, *Crassostrea virginica,* to metal-contaminated sediments. Marine Environmental Research, 48: 427–437.

Roesijadi, G., Unger, M.E., and Morris, J.E. 1988 Immunochemical quantification of metallothioneins of a marine mollusc. Canadian Journal of Fisheries and Aquatic Sciences, 45: 1257–1263.

Svendseb, C, and Weeks, J.M. 1995. The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). Marine Pollution Bulletin, 31: 139–42.

Tremblay, R., Myrand, B., and Guderley, H. 1998. Temporal variation of lysosomal capacities in relation to susceptibility of mussels, *Mytilus edulis,* to summer mortality. Marine Biology, 132: 641–649.

Viarengo, A., Ponzano, E., Dondero, F., and Fabbri, R. 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Marine Environmental Research, 44: 69–84.

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., and Koehler, A. 2007. The use of biomarkers in biomonitoring: a 2-tiered approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. Comp Biochem Phys. 146(3), 281-300.

Widdows, J., and Salkeld, P. 1992. Practical procedures for the measurement of scope for growth. MAP Technical Reports Series, 71: 147–172.

Widdows, J., and Johnson, D. 1988. Physiological energetics of *Mytilus edulis:* Scope for growth. Marine Ecology Progress Series, 46(1-3): 113–121.

Technical Annex on Supporting parameters for biological effects measurements in fish and mussels

A. Measurement of supporting metrics for fish: condition indices, GSI, HSI and age

Background

For all biological effect techniques within the OSPAR JAMP and OSPAR integrated strategy there is a requirement to report supporting parameters, and these include 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 (Gonad Somatic Index - GSI) and liver weight to whole body weight (Liver Somatic Index - LSI or Hepato Somatic Index - HSI), explanations of these are described below. Both gonad and liver weight will change markedly throughout the year and for comparative purposes these seasonal variations must be taken into account for the interpretation of biomarker responses such as EROD and VTG for example. Additionally, the condition factor (CF) is a general indicator for fish condition, similarly the condition index (CI) for mussels.

ICES WGBEC recently reviewed the measurement of these metrics and their role and importance in fish monitoring programmes, and this is described below.

Summary of supporting parameters required for fish;

Parameter	Measurement	Comment
Live fish whole body weight	To 0.1g	Blotted dry
Length of fish	To nearest mm	
Liver weight	To 0.1 g	
Gonad weight	To 0.01g	In addition record sex
Gonad length	To nearest mm	In addition record sex
Age	Conducted on otoliths	All individuals sampled

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 strong 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 the somatic body weight (Gonad Somatic Index - 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 while 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 to 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 the gonad size is the liver size relative to the somatic body weight (Liver Somatic Index – LSI, or sometimes referred to as Hepato Somatic Index - 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. gram/year) as shown in Kiceniuk and Khan, 1986; McMaster *et al*., 1991 and in Ericson *et al*., 1998, as well as the Condition Factor (CF - see reference to Foulton below) are relatively straightforward to determine and may be used as markers for adverse effects due to xenobiotic exposure. The measurement of the condition factor has not often been used in short exposure laboratory experiments, however, field observations over longer time periods indicate that it may be a valuable measure for adverse effects. (See review by van der Ost *et al*., 2003). Recent investigations related to the Fish Disease Index (WGPDMO, 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:

$$K = \text{weight} / (\text{length})^3$$

The condition factor 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 condition factor, and may be important for a number of different responses, and as such can be included in biomonitoring investigations.

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, gonadosomatic index (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.

Measurement of GSI: record whole body weight of fish and gonad weight to 2 decimal places.

*GSI = (gonad weight x 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 were 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 experiment where effect on the GSI value have been documented include petroleum mixtures (Truscott *et al*., 1983; Kiceniuk and Khan, 1986), specific PAHs (Thomas, 1988; Singh, 1989; Thomas and Budiantara, 1995), PCB mixture (Thomas, 1988), pesticides (Ram *et al*., 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 (annual) natural biological cycle it is essential that the normal background values are well known, and that the appropriate control material is used for comparison. For the GSI value it should be pointed out that during certain time times of the year the gonad development is very fast and that different GSI values are obtained only within a period of a few days/weeks. Analysis of the GSI in these time periods should be avoided. Baseline studies are important in order to evaluate suitable time 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, e.g., a state of condition when the 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 has been observed include the following species; burbot (*Lota lota*) in the northern coast of the 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 *et al*., 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 fresh water system show corresponding adverse effects (Noaksson *et al*., 2001; Noaksson *et al*., 2002). Although the above cited field investigations are not all related to suspected PAH contamination, these kinds of disorders has been created in laboratory experiment using petroleum products and a pure naphthalene (Thomas and Budiantara, 1995).

GSI Confounding factors

Although the measurement is robust and easy to perform there is a need to characterize and avoid confounding factors. For example female perch populations 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 photo period 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.

Liver size of female and/or male fish – LSI (HSI)

Liver size is measured in relation to somatic body weight, and is known as Liver Somatic Index (LSI* or HSI – see above).

Measurement of LSI: record whole body weight of fish and liver weight to two decimal places.

*LSI = (liver weight x 100) /(total body weight[#]–liver weight)

[#]subtract stomach content

LSI may be regarded as a relevant measurement since 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 vales 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; 2002) and effluent from waste water treatment plant (Kosmala *et al*., 1998).

Field situations where PAHs and/or organochlorines are suspected contaminants for increased liver size in various fish species are documented by: Sloff *et al*. (1983); Goksoyr *et al*. (1991); Kirby *et al*. (1999); Kirby *et al*. (1999); Beyer *et al*. (1996); Leadly *et al*. (1998); 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); Otto and Moon (1995); Arnold *et al*. (1995); Gadagbui and Goksoyr (1996); 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).

LSI Confounding factors

Although there is no doubt that xenobiotics could affect liver size as a result of different toxicological mechanisms it should be emphasised that, as for most biomarkers, control/reference fish should be analyzed in close/direct parallel to the exposed site(s). In addition, seasonal variation is observed in different fish species (Koivusaari *et al*., 1981; Förlin and Haux, 1990; Larsen, 1992), and must be taken into account at all times. Besides the time of the year, factors (i.e., parameters) such as feeding behaviour, gender, maturity, age, size, temperature (George *et al*., 1990), photo period, parasites, among others, needs to be taken into considerations. Baseline studies are an important strategy to finally evaluate confounding factors (Balk *et al*., 1996).

Determination of age

It is essential for the interpretation and assessment of biological effect responses that the age of 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 time periods, since the growth of a fish species may vary in different populations and at different locations, and from year to year .

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 and these include: feeding behaviour, gender, maturity, development stage, age, water temperature, presence of parasitic infections and other disease, location and seasonality.

B: Measurement of supporting metrics for mussel: condition indices

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 utilised for gametogenesis. This is manifested by a marked increase in flesh weight relative to whole body weight which increases and reaches 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, and these include mussel length, whole body weight and condition index.

Summary of 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.

Parameter	Measurement	Comment
Live whole animal weight	To 0.1g	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 0.1g	Flesh excised from open shell and drained / blotted dry
Dry flesh weight	To nearest 0.01g	80 degrees C for 24 hr and constant dry weight
Wet shell weight	To nearest 0.01g	Blotted dry
Dry shell weight	To nearest 0.01g	80 degrees C for 24 hr and constant dry weight
Internal shell volume	To 0.1ml	Not generally conducted but provides a very accurate measure of condition.

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 due to the difficulty in standardising the degree of wetness. Indices using dry flesh weight are more accurate particularly when used in relation to internal shell volume. Example of condition indices are given below;

CI “A” = 100 x Dry weight / Whole animal weight

CI “B” = 100 x Dry weight / Wet flesh weight

CI “C” = 100 x 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 post 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 Ferguson, 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 and Thiessen, 1987) and aerial exposure can adversely affect the condition of mussels.

References

Adams, S.M., Shugart, L.R., Southworth, G.R., and Hinton, D.E. 1990. Application of bioindicators in assessing the health of fish populations experiencing contaminant stress. In: McCarthy, J.F., Shugart, R.L. (Eds.). Biomarkers of Environmental Contamination. Lewis Pub., pp. 333–353.

Åkerblom, N., Olsson, K., Berg, A.H., Andersson, P.L., Tysklind, M., Förlin, L., and Norrgren, L. 2000. Archives of Environmental Contamination and Toxicology 38:225–233.

Åkerman, G., Amcoff, P., Tjärnlund., Fogelberg, K., Torrissen, O. and Balk, L. 2003. Paraquat and menadione exposure of rainbow trout (*Oncorhynchus mykiss*) –Studies of effects on the pentose-phosphate shunt and thiamine levels in liver and kidney. Chemico-Biological Interactions 142, 269–283.

Aldich, J.C. and Crowly, M. 1986. Conditions and variability in *Mytilus edulis* L. from different habitats in Ireland. Aquaculture 52: 273–286.

Andersson, T., Förlin, L., Härdig, J., and Larsson, Å. 1988. Physiological disturbances in fish living in coastal water polluted with bleached kraft pulp mill effluents. Canadian Journal of Fisheries and Aquatic Science 45: 1525–1536.

Arnold, H., Pluta, H.-J., and Braunbeck, T. 1995. Simultaneous exposure of fish to endosulfan and disulfoton *in vivo*: ultrastructural, stereological and biochemical reactions in hepatocytes of male rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicology 33: 17–43.

Bagenal, T.B. and Tesch, F.W. 1978. Age and Growth. Pages 101–136 in T.B. Bagenal, edit. Methods for assessment of fish production in fresh waters, 3rd edition. Blackwell Scientific Publications, Oxford, England.

Balk, L., Andersson, T., Förlin, L., Söderström, M., and Larsson, Å. 1993. Indications of regional and large-scale biological effects caused by bleached pulp mill effluents. Chemosphere 27: 631–650.

Balk, L., Larsson, Å. and Förlin, L. 1996. Baseline studies of biomarkers in the feral female perch (*Perca fluviatilis*) as tools in biological monitoring of anthropogenic substances. Marine Environmental Research, 42 (1–4), 203–208.

Beyer, J. 1996. Fish biomarkers in marine pollution monitoring; evaluation and validation in laboratory and field studies. Academic thesis, University of Bergen, Norway.

Celander, M., Näf, C., Broman, D., and Förlin, L. 1994. Temporal aspects of induction of hepatic cytochrome P450 1A and conjugating enzymes in the viviparous blenny (*Zoarces viviparus*) treated with petroleum hydrocarbons. Aquatic Toxicology 29: 183–196.

Dabrowski, K., Ciereszko, R.E., Ciereszko, A., Toth, G.P., Christ, S.A., El-Saidy, D., and Ottobre, J.S. 1996. Reproductive physiology of yellow perch (*Perca flavescens*): Environmental and endocrinological cues. Journal of Applied Ichtyology :12: 139–148.

Davenport, J. and Chen, X. 1987. A comparison of methods for the assessment of condition in the mussel (*Mytilus edulis* L.). J. Molluscan Stud., 53: 293–297.

Dix, T.G. and Fergussen, A. 1984. Cycles of reproduction and condition in Tasmanian blue mussel *Mytilus edulis planatus*. Aust. J. Mar Freshw. Res. 35; 307–313.Ericson, G., Lindesjöö, E. and Balk, L. (1998) "DNA adducts and histopathological lesions in perch (*Perca fluviatilis*) and northern pike (*Esox lucius*) along a polycyclic aromatic hydrocarbon gradient on the Swedish coastline of the Baltic Sea." Canadian Journal of Fisheries and Aquatic Sciences 55, 815–824.

Förlin, L., Andersson, T., Balk, L., and Larsson, Å. 1995. Biochemical and physiological effects in fish exposed to bleached kraft mill effluents. Ecotoxicology and Environmental Safety 30: 164–170.

Förlin L, Haux C. Sex differences in hepatic cytochrome P-450 monooxygenase activities in rainbow trout during an annual reproductive cycle. J Endocrinol 1990; 124: 207–213.

George S, Young P, Leaver M, and Clarke D. Activities of pollutant metabolizing and detoxification systems in the liver of the plaice, *Pleuronectes platessa*: sex and seasonal variations in non-induced fish. Comp Biochem Physiol 1990; 96C: 185-192.

Gadagbui, B.K.-M., and Goksoyr, A. 1996. CYP1A and other biomarker responses to effluents from a textile mill in the Volta river (Ghana) using caged tilapia (*Oreochromis niloticus*) and sediment-exposed mudfish (*Clarias anguillaris*). Biomarkers 1: 252–261.

Goksoyr and 10 co-authors. 1991. Environmental contaminants and biochemical responses in flatfish from the Hvaler archipelago in Norway. Archives of Environmental Contamination and Toxicology 21: 486–496.

Hodson and 8 co-authors. 1992. Effects of bleached kraft mill effluent on fish in the St. Maurice River, Quebec. Environmental Toxicology and Chemistry 11: 1635–1651.

Huuskonen, S., and Lindström-Seppä, P. 1995. Hepatic cytochrome P4501A and other biotransformation activities in perch (*Perca fluvitialis*): the effects of unbleached pulp mill effluents. Aquatic Toxicology 31: 27–41.

Janssen, P.A.H., Lambert, J.G.D., Vethaak, A.D., and Goos, H.J.Th. 1997. Environmental pollution caused elevated concentration of oestradiol and vitellogenin in the female flounder, *Platichthys flesus* (L.). Aquatic Toxicology 39: 195–214.

Johnson, L.L., Casillas, E., Collier, T.K., McCain, B.B., and Varanasi, U. 1988. Contaminant effects on ovarian development in English sole (*Parophrys vetulus*) from Puget Sound, Washington. Canadian Journal of Fisheries and Aquatic Science 45: 2133–2146.

Karels, A., Markkula, E., and Oikari, A. 2001. Reproductive, biochemical, physiological, and population responses in perch (*Perca fluviatilis* L.) and roach (*Rutilus rutilus* L.) downstream of two elemental chlorine-free pulp and paper mills. Environmental Toxicology and Chemistry 20: 1517–1527.

Kent, R.M.L. 1979. The influence of heavy infestations of *Polydora ciliate* on the flesh content of *Mytilus edulis*. J. Mar. Biol. Ass. UK. 59: 289–297.Kiceniuk, J.W., and Khan, R.A. 1987. Effect of petroleum hydrocarbons on Atlantic cod, *Gadus morhua*, following chronic exposure. Canadian Journal of Zoology 65:490–494.

Kirby, M.F., Matthiessen, P., Neall, P., Tylor, T., Allchin, C.R., Kelly, C.A., Maxwell, D.L., and Thain, J.E. 1999. Hepatic EROD activity in flounder (*Platichys flesus*) as an indicator of contaminant exposure in English estuaries. Marine Pollution Bulletin 38:676–686.

Kirby, M.F., Neall, P., and Tylor, P. 1999. EROD activity measured in flatfish from the area of the Sea Empress oil spill. Chemosphere 38: 2929–2949.

Kloepper-Sams P.J., and Owens J.W. 1993. Environmental biomarkers as indicators of chemical exposure. Journal of Hazardous Material. 35: 283–294.

Kosmala, A., Migeon, B., Flammarion, P., and Garric, J. 1998. Impact assessment of a wastewater treatment plant effluent using the fish biomarker ethoxyresorufin-O-deethylase: field and on-site experiments. Ecotoxicology and Environmental Safe 41: 19–28.

Koivusaari U, Harri M, Hanninen O. Seasonal variation of hepatic biotransformation in female and male rainbow trout, *Salmo gairdneri*. Comp Biochem Physiol, 1981; 70C: 149–157.

Larsen HE, Celander M, Goksøyr A. The cytochrome P450 system of Atlantic salmon (*Salmo salar*): II. Variations in hepatic catalytic activities and isozyme patterns during an annual reproductive cycle. Fish Physiol Biochem 1992; 10: 291–301.

Leadly, T.A., Balch, G., Metcalfe, C.D., Lazar, R., Mazak, E., Habowsky, J., Haffner, D. 1998. Chemical accumulation and toxicological stress in three brown bullhead (*Ameiurus nebulosus*) populations of the Detroit River, Michigan, USA. Environmental Toxicology Chemistry 17: 1756–1766.

Lehtinen, K.-J., Kierkegaard, A., Jakobsson, E., Wändell, A. 1990. Physiological effects in fish exposed to effluents from mills with six different bleaching processes. Ecotoxicology and Environmental Safe 19: 33–46.

Luksiene, D., Sandström, O., Lounasheimo, L. and Andersson, J. 2000. The effects of thermal effluent exposure on the gametogenesis of female fish. Journal of Fish Biology 56: 37–50.

Lutz, R.A. 1980. Mussel Culture and Harvest: A North American Perspective, Elsevier Science Publishers, B.V. Amsterdam, 305pp.

Lutz, R. A., Incze, L. S., Porter, B., and J. K. Stotz. 1980. Seasonal variation in the condition of raft-cultivated mussels (*Mytilus edulis* L.), Proc World Mariculture Society, 11, 262–268.

Newsted, J.L., and Giesy, J.P. 1993. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the epidermal growth factor receptor in hepatic plasma membranes of rainbow trout (*Oncorhynchus mykiss*) Toxicology and Applied Pharmacology 119: 41–51.

Noaksson E, Tjärnlund U, Bosveld ATC, Balk L. Evidence for endocrine disruption in perch *Perca fluviatilis* and roach *Rutilus rutilus* in a remote Swedish lake in the vicinity of a public refuse dump. Toxicol Appl Pharmacol 2001; 174: 160–176.

Noaksson, E., Linderoth, M., Bosveld, A.T.C., Norrgren, L., Zebuhr, Y. and Balk, L. 2002. "Endocrine disruption in brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump." The Science of the Total Environment, In Press.

Noaksson, E., Tjärnlund, U., Bosveld, A.T.C., and Balk, L. 2001. Evidence for endocrine disruption in perch *Perca fluviatilis* and roach *Rutilus rutilus* in a remote Swedish lake in the vicinity of a public refuse dump. Toxicology and Applied Pharmacology 174: 160–176.

Noaksson, E., Linderoth, M., Bosveld, A.T.C., Norrgren, L., Zebuhr, Y., and Balk, L. 2002. Endocrine disruption in brook trout (*Salvelinus fontinalis*) exposed to leachate from a public refuse dump." The Science of the Total Environment, In Press.

Otto, D.M.E., and Moon, T.W. 1995. 3,3´,4,4´-tetrachlorbiphenyl effects on antioxidant enzymes and glutathione status in different tissues in rainbow trout. Pharmacology and Toxicology 77: 281–287.

Pereira, J.J., Mercaldo-Allen, R., Kuropat, C., Luedke, D., and Sennefelder, G. 1993. Effect of cadmium accumulation on serum vitellogenin levels and hepatosomatic and gonadosomatic indices of winter flounder (*Pleuronectes americanus*). Archives of Environmental Contamination and Toxicology 24: 427–431.

Ram, R.N., and Sathyanesan, A.G. 1986. Effect of a mercurial fungicide on the gonadal development of the teleostean fish *Channa punctatus* (Bloch). Ecotoxicology and Environmental Safety 11: 352–360.

Rodhouse, P.G., Rhodes, C.M., Burrell, G.M., Hensey, M.P., McMahon, T., Ottway, B. and Ryan, T.H. 1984. Food resource, gametogenesis and growth of *Mytilus edulis* on the shore and in suspended culture; Killory Harbour, Ireland. J. Mar. Biol. Ass. UK: 59:289–297. Sandström O. Incomplete recovery in a coastal fish community exposed to effluent from a modernised Swedish bleached kraft mill. Can J Fish Aquat Sci 1994; 51: 2195–2205.

Sandström, O., Neuman, E., and Karås, P. 1988. Effects of bleached pulp mill effluent on growth and gonad function in Baltic coastal fish. Water Science Technolology 20: 107–118.

Sandström O, Neuman E, Thoresson G. Effects of temperature on life history variables in perch, *Perca fluviatilis*. J Fish Biol 1995; 47: 652–670.

Singh, H. 1989. Interactions of xenobiotics with reproductive endocrine functions in a Protogynous teleost, *Monopterus albus*. Marine Environmental Research 28: 285–289.

Sloff W., van Kreijl, C.F., Baars, A.J. 1983. Relative liver weights and xenobiotic-metabolizing enzymes of fish from polluted surface waters in the Netherlands. Aquatic Toxicology: 4: 1–14.

Stentiford, G.D., Bignell, J.P., Lyons, B.P., Thain, J.E. and Feist, S.W. 2010. Effect of age on liver pathology and other diseases in flatfish: implications for assessment of marine ecological health status. Marine Ecology Progress Series: 411: 215–230.

Stephensen, E., Svavarsson, J., Sturve, J., Ericson, G., Adolfsson-Erici, M., and Förlin. 2000. Biochemical indicators of pollution exposure in shorthorn sculpin (*Myoxocephalus scorpius*), caught in four harbours on the southwest coast of Iceland. Aquatic Toxicology: 48: 431–442.

Thiessen, B.FR. 1987. Infestation of *Mytilus edulis* by *Mytilicola intestinalis*. Ophelia 27: 77–86.

Thomas, P. 1988. Reproductive endocrine function in female Atlantic croaker exposed to pollutants. Marine Environmental Research 24: 179–183.

Thomas, P., and Budiantara, L. 1995. Reproductive life history stages sensitive to oil and naphthalene in Atlantic croaker. Marine Environmental Research 39: 147–150.

Truscott, B., Walsh, J.M., Burton, M.P., Payne, J.F., and Idler, D.R. 1983. Effect of acute exposure to crude petroleum on some reproductive hormones in salmon and flounder. Comparative Biochemistry and Physiology 75C: 121–130.

Tjärnlund, U., Ericson, G., Lindesjöö, E., Petterson, I., Åkerman, G. and Balk, L. 1996. Further studies of exhaust from two-stroke outboard engines on fish. Marine Environmental Research, 42: 267–271.

Vallin, L., Nissling, A., and Westin, L. 1999. Potential factors influencing reproductive success of Baltic cod, *Gadus morhua*: a review. Ambio 28: 92–99.

van der Oost, R., Beyer, J., and Vermeulen, N.P.E. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13: 57–149.

WGPDMO. 2011. Report of the ICES Working Group on Pathology and Diseases of Marine Organisms. International Council for the Exploration of the Sea, Copenhagen.

Technical Annex on Assessment criteria for biological effects measurements

Table 1. Assessment criteria for biological effects measurements. Values are given for both background assessment levels (BAC) and environmental assessment criteria (EAC), as available.

Biological Effect	Applicable to:	BAC	EAC
VTG in plasma; μg/ml	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 protein pmol/min/ mg protein S9 * pmol/min/ mg 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) ng/ml; HPLC-F (2) pyrene-type μg/ml; synchronous scan fluorescence 341/383 nm (3) ng/g GC/MS * 1-OH pyrene ** 1-OH phenanthrene	Dab	16 (1) * 3.7 (1) ** 0.15 (2)	22(2)
	Cod	21 (1) * 2.7 (1) ** 1.1 (2)	483 (3) * 528 (3) ** 35 (2)
	Flounder	16 (1) * 3.7 (1) ** 1.3 (2)	29(2)
	Haddock	13 (1) * 0.8 (1) ** 1.9 (2)	35(2)
DR-Luc; ng TEQ/kg dry wt, silica clean up	Sediment (extracts)	10	40
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; minutes	Cytochemical; all species	20	10
	Neutral Red Retention: all species	120	50
Micronuclei; $^{0}/_{00}$ (frequency of micronucleated cells) [1] Gill cells [2] Haemocytes [3] Erythrocytes	*Mytilus edulis*	2.5 [1] 2.5 [2]	
	Mytilus galloprovincialis	3.9 [2]	
	Mytilus trossulus	4.5 [2]	
	Flounder	0.0-0.3 [3]	
	Dab	0.5 [3]	
	Zoarces viviparus	0.3-0.4 [3]	
	Cod	0.4 [3]	
	Red mullet	0.3 [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 prot^{-1} [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+] 75 [3++]	109 [2+] 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 NA: Not applied	Dab	Fish Disease Index (FDI): F: *1.32*, 0.216 M: *0.96*, 0.232 F: *1.03*, 0.349 M: *1.17*, 0.342 F: *1.09*, 0.414 M*: 1.18*, 0.398 M: males F: females	Fish Disease Index (FDI): F: *NA*, 54.0 M: *NA*, 47.7 F: *50.6*, 19.2 M: *38.8*, 16.1 F: *48.3*, 21.9 M: *35.2*, 16.5
Liver histopathology-non specific	Dab	NA	Statistically significant increase in mean FDI level in the assessment period compared to a prior observation period *or* Statistically significant upward trend in mean FDI level in

			the assessment period
Liver histopathology- contaminant-specific	Dab	Mean FDI <2	Mean FDI ≥ 2 A value of 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 Flounder Cod Red mullet *Zoarces viviaprus*	5	
Scope for growth	Mussel (*Mytilus* sp.)	[3]	[3]

[3] BAC and EAC values have yet to be determined for Scope for growth.

Joules/hr/g dry wt.	(provisional, further validation required)		
Hepatic metallothionein µg/g (w.w.) [1] Whole animal [2]Digestive gland [3]Gills * Differential pulse polarography	*Mussel edulis*	0.6 [1*] 2.0 [2*] 0.6 [3*]	
	Mytilus galloprovincialis	2.0 [1*] 3.9 [2*] 0.6 [3*]	
Histopathology in mussels	VVbas: Cell type composition of digestive gland epithelium; $\mu m^3/\mu m^3$ *(quantitative)*	0.12	0.18
	MLR/MET: Digestive tubule epithelial atrophy and thinning; µm/µm *(quantitative)*	0.7	1.6
	VVLYS & Lysosomal enlargement; $\mu m^3/\mu m^3$ *(quantitative)*	VvLYS 0.0002	V>0.0004
	S/VLYS: $\mu m^2/\mu m^3$	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*). Abbreviations used: 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 and 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&P Norge & 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.

Technical Annex on Protocols for extraction, clean-up and solvent exchange methods for small-scale bioassays

1. Introduction

The aims of this document are as follows:

To produce standardised protocols for bioassay extractions;

To enhance consistency of applications between laboratories;

To ensure applicability throughout OSPAR maritime area, including in estuarine waters;

To ensure comparability of reported data for assessment purposes.

History:

This document has been developed from a previous review, and relates particularly to background documents on water and sediment bioassays and *in vitro* bioassays prepared by ICES expert groups WGBEC and SGIMC. This paper describes a recommended methodology for extraction protocols for use of small scale *in vitro* and *in vivo* bioassays

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, 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).

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 that will be used, differences in extraction solvent and, in particular, sample cleanup (Section 2.5), may be applied.

Klamer *et al*., 2005, 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, waste water, sediment pore water, potable water, rain, snow, ice.

Before detailed protocols are presented, the basic layout of each extraction and cleanup protocol is given below.

Solid samples

Protocol steps	Comment
1. Sample preparation	Sample sieved when necessary (e.g., sediment), dried and homogenised.
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 co-extracted 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. Cleanup of crude extract	Gel Permeation Chromatography (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. Pre-treatment 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 pre-treated sample	pH treatment with 100 µl 1N HCl, extraction with 2 mL ethyl acetate.
4. Cleanup 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 N2, solvent exchange into 50 µl DMSO.

2.1 Protocol for extraction of dried, solid samples with Accelerated Solvent Extraction (5 g sample). Steps are numbered S.1, S.2, etc.

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 gram 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	Temp	Preheat time	Static time	Flush volume	Purge time	Static Cycles
	[psi]	[°C]	[min]	[min]	[ml]	[sec]	
Hexane/Acetone 9:1 v:v	2000	100	5	5	60	90	3
DCM or DCM/modifier**	2000	45–100*	5	5	60	90	1–3*

* Set temperature to 45 to 50°C and # of cycles to 3 for use with ER-CALUX and similar tests.

** methanol or acetone.

S.5. If water is co-extracted, dry the extract using anhydrous sodium sulphate. 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 (Section 3.3) or store the crude extract at -20°C until further use.

2.2 Protocol for extraction of aqueous samples with Solid Phase Extraction devices. Steps are numbered A.1, A.2, etc.

Extraction

A.1. Assemble the SPE cartridge. For samples up to 20L, 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 100L samples, a multi column 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 multi column 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 the samples have 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.

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 ml, 4 ml and 4 ml DCM respectively.

A.11. Remove the tube from under each column and replace it with a clean one. Repeat Section 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 l 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 sulphate. 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 e.g., 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 (see paragraph 3.3). Transfer sample into a glass Store extracts in freezer at -20°C. Samples can be stored for a maximum of one year.

2.3 Protocol for extraction of fish bile samples. Steps are numbered B.1, B.2, etc.

The extraction procedure described below is taken from the work by Legler *et al*., 2002.

Extraction

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 40U of β-glucuronidase–arylsulfatase (from *H. pomatia*).

B.4 Incubate tubes overnight (17–18 h) in a water bath (37°C, gentle shaking).

3. Cleanup

3.1 Broad-spectrum clean-up

Cleanup procedures are applicable to all crude extracts. However: the user has to choose between two fundamentally different cleanup principles: *broad spectrum* or *target* cleanup.

Gel Permeation Chromatography 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 upon electronic interaction between the column material and the extracted compound. This secondary mechanism is used for removal of molecular sulphur (as S8) from the crude extract, using DCM as eluting solvent. GPC cleanup requires careful calibration using a series of different compounds. This type of cleanup has successfully been applied to very different *in vitro* bioassays: Microtox, Mutatox, (anti)DR-CALUX, (anti)ER-CALUX, *umu*-C (e.g., by Klamer *et al*., 2005 and Houtman *et al*., 2004).

C.1. Set up of GPC equipment. For semi-preparative cleanup, large-diameter columns may be used in series, e.g. polystyrene-diphenylbenzene copolymer columns (PL-gel, 5 or 10µm, 50Å, 300x25 mm or 600x7.5 mm, preferably in a thermostatic housing at 18ºC, with a PL-gel pre-column 5 or 10 µm 50x7.5mm). Use an HPLC pump with 10 ml/min 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) 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 sulphur (as S8) elutes (elution of the extract is monitored using a UV detector at 254 nm.) This range, however, should be carefully monitored using a 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 200–2000 µL batches, 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 (see paragraph 3.3) or store at -20ºC until further use.

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, $AgNO_3$).

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 cleanup is as follows:

TBA sulphite solution

Wash a 250 ml separation-funnel with hexane, fill the funnel with 100 ml HPLC water and dissolve 3.39 grams TBA

Rinse the solution three times with 20 ml hexane.

Dissolve 25 gram sodium sulphite in the washed solution.

Store the solution in a dark bottle (Maximum storage time 1 to 2 weeks).

Sulphur clean-up

Add 2.0 ml TBA-sulphite solution and 2.0 ml isopropanol to the extract, mix for 1 minute on a vortex. Sulfur clean-up is complete if precipitation is visible. Add an extra 100 mg sodium sulphite if no precipitation is present and mix during 1 minute on a vortex. Repeat the addition if necessary.

Add 5 ml of HPLC-grade water, mix for 1 minute on a vortex.

Let the layers separate during approximately 5 minutes, transfer the hexane layer to a clean collection vial.

Add 1 ml hexane to the extract and mix during 1 minute on the vortex. Let the layers separate layers en 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

Prepare a solution of hexane/diethylether (97/3; v/v)

Place a small piece of glass wool in a separation. As the performance of the following steps is column-dependent (see Annex 1 for column layout).

Fill the column with 5 grams of 33% silica and tremble the cells with the engraver pen. Add 5 grams of 20% silica and tremble the column once more. Add a small amount of dried sodium sulphate to the top of the column.

Elute the column with 20 ml hexane/diethylether solution.

Bring the extract on the column as soon as the meniscus reaches the sodium sulphate. Wash the collection vial of the extract twice with approximately 1 ml hexane/diethylether solution.

Place a clean collection vial under the column and elute the column with 38 ml hexane/diethylether.

Evaporate the hexane until less than 1 ml is left.

Proceed to solvent exchange (see below, 3.3)

ER CALUX

This section describes the cleanup of deconjugated fish bile extract for use in the ER-CALUX assay. Steps are numbered B5, B6, 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 B6 and B7 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 (see below, 3.3).

3.3 Solvent exchange

Klamer and van Loon (1998) and Bakker *et al*. (2007) developed criteria and evaluated co-solvents for bioassays. The ideal co-solvent or carrier solvent used for ecotoxicity testing should meet the following criteria: (1) effective: sufficiently high solubility of target compounds, (2) water-miscible: the carrier solvent must be water-miscible, and (3) 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 co-solvent.

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 minus 20^{o}C degrees C until bioassayed, and should not be stored for longer than twelve weeks.

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 minutes or placed in the ultrasonic bath for ten minutes 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 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 enable the calculation of the NOEC, LOEC and EC_{50} values with greater precision.

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).

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 degrees 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 hr 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.

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 shown below:

	Test organism	Test volume (ml)	Number of organisms/cells per test vessel	Reference
In vivo	Mussel embryo	1–5	50 per ml	ASTM724
	Oyster embryo	1–5	50 per ml	ASTM724
	Sea urchin	1–5	40 per ml	ASTM1563
	Microalgae (freshwater and seawater)	1–5	5x106 cells /L	ISO8692, ISO10253
	Macrophyte germination	1–5	500-1000 zygotes per ml	Brooks *et al*., 2008
	Daphnia	1–5	1 per test vessel	ISO6341
	Acartia / Nitocra	5	5 per test vessel	ISO 14669
	Tisbe	5	5 per test vessel	ISO14669
	Fish embryo	2–5 ml	1 per 2ml test vessel	OECD draft guideline
In vitro	YES, YAS, anti-YES, anti-YAS	200 µl	0.8 x 106 cells/ ml	Tollefsen *et al*., 2007
	ER calux	200 µl	5-10 x 105cells/ml	Legler *et al*., 2003
	Primary cell cultures	200 µl	5x105 cells/ml	Tollefsen *et al*., 2003
	Cell lines	200 µl	5-10 x 105cells/ml	

Matrix	Procedure	Bio-assay	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.

6. References

Bakker, J.F., Bulzunce-Segarra, M.J., Castro, R., van de Heuvel-Greve, M., Klamer, H.J.C., Brack, W., Altenburger, R., Poulsen, V., Thomas, K.V., Leonards, P.E.G.. 2007. Effect directed analysis and toxicity identification evaluation. In: Sediment quality and impact assessment of pollutants. Sustainable Management of Sediment Resources. Chapter 5. D. Barceló, and M. Petrovic (Eds.), Elsevier, Amsterdam, 333 pp., ISBN: 9780-444-51962-7.

Besselink, H.T., Schipper, C.A., Klamer, H.J.C., Leonards, P.E.G., Verhaar, H.J.M., Felzel, E.C., Murk, A.J., Thain, J.E., Hosoe, K., Schoeters, G., Legler, J., and Brouwer, A. 2004. Intra- and interlaboratory calibration of the DR CALUX (T) bioassay for the analysis of dioxins and dioxin-like chemicals in sediments. *Environ. Toxicol. Chem.* 23(12), 2781–2789.

Houtman, C.J., Cenijn, P.H., Hamers, T., Lamoree, M.H., Legler, J., Murk, A.J. and Brouwer, A. 2004. Toxicological profiling of sediments using in vitro bioassays, with emphasis on endocrine disruption. *Environ. Toxicol. Chem.* 23(1), 32–-40.

Houtman C.J., Leonards, P.E.G., Kapiteijn, W., Bakker, J.F., Brouwer, A., Lamoree, M.H., Legler, J., Klamer, H.J.C. 2007. Sample preparation method for the ER-CALUX bioassay screening of (xeno-)estrogenic activity in sediment extracts. Marine Pollution Bulletin, 54.

ISO. 1999. ISO 14669 Water Quality - Determination of acute lethal toxicity to marine copepods (*Copepoda*, *Crustacea*).

Klamer, H.J.C., Thomas, K., Tollefsen, K-E. 2005. *Extraction procedures applicable to small-scale bioassays*. Discussion paper, Working Group on Biological Effects of Contaminants.

Klamer, H.J.C., Leonards, P.E.G., Lamoree, M.H., Villerius, L.A., Åkerman, J.E., Bakker, J.F. 2005. A chemical and toxicological profile of Dutch North sea surface sediments. *Chemosphere* 58, 1579–1587.

Legler, J., Leonards, P., Spenkelink, A., Murk, A.J. 2003. *In vitro* biomonitoring in polar extracts of solid phase matrices reveals the presence of unknown compounds with estrogenic activity. Ecotoxicology, 12: 239–249.

Legler, J., Jonas, A., Lahr, J., Vethaak, A.D., Brouwer, A. and Murk, A.J. 2002. Biological measurement of estrogenic activity in urine and bile conjugates with the *in vitro* ER-CALUX reporter gene assay. *Environ. Toxicol. Chem.* 21(3), 473–479.

Leonards, P.E.G., van de Veen, I., Lohman, M., Felzel E. and Man, S. 2002. *Comparison of extraction and clean-up methods for use with the DR-CALUX assay*. RIVO, report C048/02, (*in Dutch*).

Tollefsen, K.-E., Mathisen, R. and Stenersen, J. 2003. Induction of vitellogenin synthesis in an Atlantic salmon (*Salmo salar*) hepatocyte culture: a sensitive *in vitro* bioassay for the (anti)estrogenic activity of chemicals. *Biomarkers*. 8, 394–407.

Tollefsen K-E., Harman, C., Smith, A., Thomas, K.V., 2007. Estrogen receptor (ER) agonists and androgen receptor (AR) antagonists in effluents from Norwegian North Sea oil production platforms. Original Research Article. Science of the total environment, 386: 134–144.

Thomas, K.V., Balaam, J., Hurst, M., Nedyalkova, Z. and Mekenyan, O. 2004. Potency and characterization of estrogen-receptor agonists in United Kingdom estuarine sediments. Environ. Toxicol. Chem. 23(2), 471–479.

Discussion document on survey design for integrated chemical and biological effects monitoring

Background

The joint OSPAR/ICES Workshop on Integrated Monitoring of Contaminants and their Effects in Coastal and Open Sea Areas (WKIMON) were requested 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 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 re-designing their station network.

Sampling for a single parameter or for integrated monitoring both 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 in section ‘Some statistical considerations in integrated monitoring’.

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 ’UK approach to survey redesign’ below). 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 sub-regions) as a core element of its assessment system. In anticipation of this, the Regional Conventions (OSPAR, HELCOM, MEDPOL *etc*.) are developing proposals for the definition of sub-regions within their areas.

SGIMC considered, therefore, that the development of survey design, including sub-regions 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., It will be necessary for the MSFD 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 North East Atlantic.

In the light of the current implementation for MSFD and the availability of statistical advice from the relevant environmental WGs in ICES, SGIMC are of the opinion that it is not possible for them to progress this question of survey design further at this time. SGIMC recommend that this discussion document be forward

to OSPAR as a contribution to the wider survey design task in relation to harmonisation of OSPAR and MSFD programmes.

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 as long as 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 one another 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, though the latter is also driven by other considerations (inside / outside the spawning period, *etc*.).

Survey design: general

Survey design is driven by the objectives of the sampling, which are (WKIMON III report, p. 170):

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

Of course, each choice of sampling points and sample sizes for a survey leads to some data about marine contamination and possible effects (as long as 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 to / higher than at position B) or
- a temporal comparison (level at position A at time T1 was lower than / comparable to / higher than the level at this position at time T2)
- 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, which is optimal in the sense that with a pre-specified effort the most precise map of the spatial parameter distribution is obtained or that a pre-specified 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 1below. 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 last 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 due to practical restrictions.

Survey design: optimal design for fixed stations

Table 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 ß. Must be specified for each parameter.	no standard
1 - ß, 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

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 7.1 above).

Step 2: Determine the necessary number of replicates per sampling location (needs knowledge about 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 in 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 4 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 means square error 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.

Survey design: a first approach for a fixed stations 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 such 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.

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 1 shows an example of this, for the Region defined as Humber/Wash. The region consists several strata, which include Water Framework Directive water bodies in the 0 – 1 nautical mile limit, an intermediate stratum 1 – 12 nautical mile 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 due to 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.

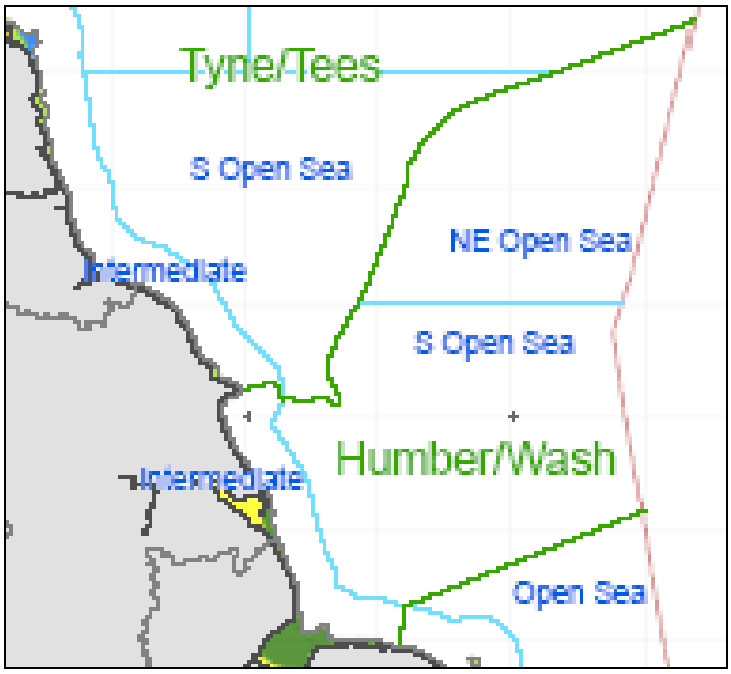


Figure 1 An example of the UK regional approach to redesigning the national monitoring station network.

Sample size for integrated monitoring

JAMP Guidelines specify sample sizes for each parameter, though without explicit justification in terms of error probabilities and detectable effects. The sizes given by JAMP seem mainly to be guided by practical considerations (get enough material for analysis and considering the time available to collect it). Additional to these considerations, a formal sample size calculation for single parameters could be done by using Background Assessment Concentration (BACs), Environmental Assessment Criteria (EACs), knowledge of analytical errors and standard specifications for acceptable error probabilities, in order to ensure that categorizations of parameters in for example 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) due to 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 parameters errors would be used for sample size calculation in the usual way. However, at present such a mathematical form is not yet available.

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