OSPAR COMMISSION

Final Report of the Study Group on developing new guidelines for the monitoring of biological EFFects of contaminants (SGEFF)

A joint OSPAR/HELCOM Study Group established under HASEC 22/13/1, Annex 5

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Background

This is the report of the Study Group on developing new guidelines for the monitoring of biological EFFects of contaminants (SGEFF), established by OSPAR and HELCOM in 2022. The group has consisted of 22 experts representing organisations and countries from both regions, which has ensured that any differences between the regions were taken into consideration. The aim was to recommend an updated monitoring programme with harmonised guidelines suitable to be implemented in both sea regions.

The group was asked to address the following:
Step 1: To review and list (update) a "core set" of relevant parameters
- To consider the existing guidelines as the fundamental basis of the new guidelines in sediment, water and biota
- To reassess the existing biomarkers and bioassay protocols already published in ICES TIMES series and suggest new developments (analyses, EAC/BAC, new supporting parameters)

Step 2: To update the sampling strategy
- To review the current sentinel species and to consider new ones
- To review current sampling recommendations regarding, e.g., spatial coverage and frequency

Step 3: To optimize the current integrated biological effects approaches in use and to propose new ones
- To consider the new developments in ecological risk assessment (e.g., effect-based methods [EBM] and effect-directed-analysis [EDA]) for potential integrated chemical-biological monitoring application at the regional and sub-regional levels

Step 4: To review the current spatiotemporal assessment procedures in each country and to elaborate these according to different monitoring scenarios
Step 5: To consider a new quality assurance programme (after the BEQUALM)
Step 6: Finalising the new guidelines and their evaluation by the Regional Commissions

Throughout the work with this report, the Study Group has been focused on adapting the recommendations to existing chemical and biological effects monitoring programmes in OSPAR and HELCOM countries. Recommended methods can be found in section 1, species selection is in section 2, assessment criteria and quality assurance in section 3, recommendations for monitoring design are described in section 4, a discussion of effect-based monitoring (EBM) and effect-directed analyses (EDA) in section 5 and correspondence with current national guidelines can be found in section 6.

Developing guidelines for chemical monitoring has not been within the remit of SGEFF, but the report highlights the need for chemical analyses in the context of biological effect monitoring (section 4). The motivation for using biological effects in monitoring was reviewed recently in an ICES viewpoint (Hylland et al., 2021), and this report was used as a background for the work reported here. Biological effects in monitoring have matured

substantially since the early OSPAR guidelines in the 1990s, particularly as concerns quality assurance and assessment (sections 3 and 5). Many of the biomarkers from earlier guidelines have been retained, although methods and approaches have been refined. More common than not, mechanisms covered by existing biomarkers are actually highlighted in studies using emergent technologies, i.e. transcriptomics and proteomics.

The design of monitoring programmes for biological effect samples will largely align with similar programmes for chemical monitoring, allowing for efficient coordination with minimal effort. This strategy has already been adopted in many existing programmes. This is also clearly appropriate to reduce the number of organisms sampled.

1. Recommended biomarkers and bioassays

Effect-oriented monitoring was introduced in the 1980s in some European countries and more widely in the 1990s following recommendations from OSPAR and subsequent development of guidelines (OSPAR 1997, 1998). There has been significant scientific progress since then, both in terms of method development and not least in our understanding of biological effect methods application and interpretation. The current document retains the concepts and many methods from earlier guidelines, but has been further progressed, incorporating the framework developed by ICES/OSPAR working groups (reported in Davies & Vethaak, 2012) and HELCOM activity (CORESET, 2013).

Biomarkers are rarely used in isolation, as there are typically multiple contaminants present in any given environment, and there is a need to identify and quantify a broad range of responses. Such a set of biomarkers are commonly referred to as a "battery" of biomarkers or as a "multibiomarker approach" for biomonitoring (Lehtonen et al., 2006; Schiedek et al., 2006). The biomarkers recommended here reflect biological responses associated with contaminant-related toxicity and are linked to potential ecosystem consequences (Hylland et al., 2021). These biomarkers have been documented to be specific to contaminant exposure and were selected from a broader list of potential biomarkers (section 1.1, Appendix A).

Bioassays are laboratory-based methods used to assess the toxicity of environmental matrices such as water and sediment (or its extracts) under controlled conditions (see Section 1.2). These include both *in vitro* cell-based and *in vivo* bioassays, which indicate relevant biological responses for assessing organismal and ecosystem health. Bioassays encompass whole-cell and reporter gene assays, tests using unicellular and small organisms, as well as enzyme and receptor-binding assays.

The term *effect-based monitoring (EBM)* can refer to either a program using biomarkers in organisms sampled from the environment or an approach that involves testing extracts from water or sediment with bioassays. A complementary approach, *effect-directed analysis (EDA)*, enhances toxicity assessments by chemically fractionating environmental extracts and testing individual fractions to identify specific toxic components. EBM and EDA play crucial roles in linking chemical contamination to biological effects and are further discussed in Section 5.

1.1 Biomarkers

Over the past decade, there has been an increasing focus on mechanisms of toxicity in environmental monitoring, mainly because some mechanisms directly link to decreased health status of organisms potentially causing population impacts and consequences for the ecosystem (Hylland et al., 2021).

Criteria for appropriate biomarkers to be considered for marine effect-monitoring have been discussed in the International Council for the Exploration of the Sea's (ICES) Working Group on Biological Effects of Contaminants (WGBEC) and in HELCOM (Coreset, 2013) over the

past few decades. These properties have been adapted from ICES WGBEC (2010) and Hylland et al. (2017a):

(i) The method should be able to separate contaminant-related effects from natural processes

(ii) There should be some knowledge of dose-response of the method in relation to contaminant exposure

(iii) The mechanism of toxicity for the method should be understood

(iv) Mechanisms for quality assurance for the method need to be established

(v) Assessment criteria must be available for responses in selected species to ensure comparability of results between different geographical regions

Biomarkers are natural processes in organisms that are strongly affected by exposure to contaminants. This also means they may be affected by other factors, i.e. other environmental factors. As is implied in (i) above, such confounders are well-known for the biomarkers recommended below and, where relevant, can be corrected for through sampling design (e.g. seasonal differences or differences between female and male).

The above criteria exclude many methods as there is a requirement for extensive knowledge of the biomarker, including clear evidence for a relationship between contaminant exposure and response in both laboratory exposures and field studies. Additionally, there are requirements for assessment criteria and quality assurance, which are not readily available for novel methods. Motivation for the methods recommended herein can be found below.

The focus in this report has been to develop guidelines that incorporate biological effect methods that will indicate potential ecosystem consequences across trophic levels. Methods that have been included in earlier programmes, but have not been recommended here, can be found in Annex A (HASEC 25/05/04Add2), including description and motivation.

The recommended biomarkers for effect-based monitoring in OSPAR and HELCOM are presented in Table 1. As shown in the table, most of the biomarkers have already been recommended or used in different contexts, such as OSPAR guidelines, HELCOM guidelines, in the ICES/OSPAR SGIMC framework (OSPAR 2013, Hylland et al., 2017a, Vethaak et al., 2017). They have all been recommended for use in monitoring by the ICES Working Group on Biological Effects of Contaminants (WGBEC) and many in HELCOM (Coreset, 2013).

Table 1. Recommended biomarkers, inclusion in earlier guidelines and organism. ICES TIMES protocols are available and all methods have been recommended by ICES WGBEC. SGIMC signifies the concluding work group for the ICES/OSPAR framework for integrated effect-based contaminant monitoring in 2013. HELCOM recommendations are from Coreset (2013).

Toxicological process	Biological effect method (biomarker)	J	H	S	Organism
Carcinogenesis	Neoplasia or tumours in lives	X			
Endocrine Disruption	Vitellogenin in plasma		X	X	
Genotoxicity	Micronucleus frequency in erythrocytes			X	
	DNA strand breaks in erythrocytes			X	Fish
Modulation of biotransformation	Cytochrome P4501A activity (EROD) in liver	X		X	
Neurotoxicity	AChE activity in muscle			X	
Genotoxicity	Micronucleus frequency in hemocytes			X	
Membrane toxicity	Membrane integrity in digestive gland	X		X	Bivalve
Metabolic impairment	AChE activity in foot or gill		X	X	
Developmental toxicity	Embryo malformation		X		Amphipod
Endocrine disruption	Imposex/intersex indicators	X	X	X	Gastropod

J: OSPAR JAMP/preCEMP/CEMP; H: HELCOM; S: SGIMC (OSPAR, 2013)

Biological effect methods that are part of time-series of monitoring need to be considered, to maintain consistent temporal assessments. Two of biomarkers recommended (carcinogenesis and developmental toxicity) reflect large-scale changes or direct population-related impacts, so there is limited direct, experimental data confirming all points above. Both have however been shown contaminant-specificity and are valuable precisely because they reflect contaminant effects on a large scale or directly on natural populations.

Metabolites of polycyclic aromatic hydrocarbons (PAHs), measured in bile are exposure markers; their description is included in section 4. PAH metabolites are considered an important component of effect-based monitoring programmes due to the ubiquitous nature of PAH exposure.

Below is a brief description of the above recommended biomarkers in fish, bivalves, amphipods and gastropods along with a rationale for their selection.

Recommendation - selection of biomarkers

The biomarkers indicated in Table 1 cover different mechanisms of toxicity, will respond to different contaminants and represent different trophic levels. While it is clearly optimal to include all in an effect-based monitoring programme, there may be national and regional reasons to implement a selection of the methods. All the tissue-based methods can be established in any European biochemistry laboratory and have low analytical costs (tens of €). They can be directly integrated in on-going programmes for chemical monitoring, if relevant. Microscopy-based methods (neoplasia, micronucleus, embryo aberrations, imposex/intersex) can also be established in any European biology laboratory but have somewhat varying time-requirements.

The final selection of biomarkers to be implemented in the national monitoring programme for any country should allow consideration of ongoing programmes, but at least five of the recommended biomarkers should be included to monitor different modes of toxicity and to ensure sufficient protection of the marine environment.

In addition, bile metabolites of PAHs should be included due to the ubiquitous nature of PAHs in marine ecosystems and uncertainty in fish tissue chemical analyses due to active biotransformation.

Fish: biomarkers

Carcinogenesis

Hepatic neoplastic lesions have previously been linked to the presence of hazardous chemicals in the marine environment, owing to the pivotal role undertaken by the liver in the transformation and detoxification of hazardous chemicals and their metabolites (Hinton, 2001). Numerous field studies, particularly those undertaken in North America spanning approximately 25-years, have identified polycyclic aromatic hydrocarbons (PAHs), and to a lesser extent polychlorinated biphenyls (PCBs), as a causative factor for carcinogenesis in several fish species (Myers et al., 2003). High incidences of hepatic neoplasms also affect the common dab (*Limanda limanda*) in Northeast Atlantic offshore regions, with lower prevalence also reported in European flounder (*Platichthys flesus*) from estuarine and coastal regions of the North, Irish and Baltic Seas (Stentiford et al., 2003; Lang et al., 2006). Elsewhere, laboratory exposures using small fish models have also demonstrated preliminary evidence of links between chemicals (including PAHs) and carcinogenesis (Hawkins et al., 1985; Larcher et al., 2014). Determination of causality in field and laboratory studies is challenging since cancer is a chronic disease that manifests over long periods following exposure. Age can also be a confounding factor regarding the development of hepatic neoplastic lesions, whereby high incidences are generally seen in older populations.

Accordingly, age determination is an important consideration when undertaking regional assessments.

Despite these challenges, hepatic neoplasms remain an important indicator of environmental consequences of contaminants, since it represents a final step following a sequence of causal events occurring at different levels of organization e.g. subcellular, cellular, tissue. Carcinogenesis remains recommended as a biomarker of contaminant exposure for environmental health assessments.

Endocrine disruption

Vitellogenin (Vtg), the female egg-yolk precursor, is a sensitive biomarker in the plasma of male fish for detecting oestrogen exposure. While mature female fish naturally produce Vtg and incorporate it into their eggs, male or juvenile fish liver can produce Vtg in response to (xeno)estrogen exposure. The protein then accumulates in the blood, where it can be measured. Several endocrine disrupting chemicals (EDCs) can cause Vtg induction, including natural hormones (e.g. 17β-oestradiol and estrone), synthetic hormones such as 17α-ethinylestradiol (EE2) and other EDCs like bisphenol A (BPA) (Baynes et al., 2023).

Genotoxicity - micronucleus frequency

The micronucleus (MN) assay is a marker for breakage or loss of chromosomes. These can form during cell division as the result of different processes, either due to altered chromosome segregation, during processing of DNA strand breaks in the cell, with loss of acentric fragments in mitosis or during apoptosis. Some compounds may act as genotoxins or cytotoxins and induce the MN frequency (Al-Sabti & Metcalfe 1995; Baršienė et al., 2004; Dolcetti and Vernier, 2002).

It is important to establish MN baseline levels in the species used for biomonitoring (Stankevičiūtė et al., 2022). MNs and other nuclear abnormalities have been quantified in various marine and brackish water fish species, e.g. cod (*Gadus morhua*), dab (*Limanda limanda*), European flounder (*Platychthys flesus*), eelpout (*Zoarces viviparus*), perch (*Perca fluviatilis*), red mullet (*Mullus barbatus*), common sole (*Solea solea*) and flathead grey mullet (*Mugil cephalus*) (Roubeix et al., 2023; Martinez-Gómez et al., 2017; Hansson et al., 2014; Benedetti et al., 2012; Piva et al., 2011; Hylland et al., 2008).

Genotoxicity - DNA strand breaks in blood cells

DNA strand breaks using the comet assay has been widely used in human health studies and is increasingly used in marine ecotoxicology.

It is widely established that exposure to a range of chemicals may lead to increased intracellular levels of radicals, which in turn may lead to damage to DNA, expressed as strand breaks. The method requires cells in suspension, so is most commonly used with circulating cells, e.g. red or white blood cells. DNA strand breaks are continuously generated

in all cells, and it is therefore crucial that appropriate baseline levels are set for the relevant species and tissue (Wessel et al., 2010).

Modulation of biotransformation

Cytochrome P450 1A (CYP1A) is involved in the metabolism of planar compounds including polychlorinated biphenyls (PCBs), halogenated dioxins and PAHs (Goksøyr & Förlin, 1992). The method is arguably the most widely used marine biomarker. An induction of EROD activity is used as an indicator of exposure to compounds that can act as ligands for the aryl hydrocarbon receptor (AhR) (Goksøyr, 1995).

Hepatic cytochrome P4501A activity has been used in monitoring with a range of species, including cod, flounder, sole, dab and eelpout (e.g. Franco et al., 2022; Kopko & Dabrowska, 2018; Díaz-Garduño et al., 2018; Sturve et al., 2017; Asker et al., 2016; Dévier et al., 2013; Monteiro et al., 2007; Richardson et al., 2001). EROD is sex-specific in some species and season-dependent, as it will be affected by reproductive processes in many species. The biomarker may be inhibited by other contaminants such as organotins and 2-3 ring PAHs, but its sensitivity to important environmentally relevant contaminants still merits its inclusion in the recommendation from SGEFF.

Neurotoxicity

Acetylcholinesterase (AChE) functions in the degradation of the neurotransmitter acetylcholine, a neurotransmitter, regulating the amount of acetylcholine present in postsynaptic neuromuscular junctions. When AChE is inhibited, it cannot decrease the acetylcholine concentration, causing continued stimulation of muscle/nerve fibres (Bocquene & Galgani, 1998).

Acetylcholinesterase (AChE) maintains acetylcholine signalling in the nervous system of all vertebrates and some invertebrates (but see discussion of AChE in bivalves below). Inhibition of acetylcholinesterase activity is a neurotoxic response, which is strongly affected by exposure to chemicals such as carbamates and organophosphate pesticides (e.g. Augusti-Tocco et al., 2006; Fulton & Key, 2001; Grandjean & Landrigan, 2014).

Activity and basal levels vary between fish species, with e.g. flounder having higher activity/basal levels compared to dab (Roubeix et al., 2023; Burgeot et al., 2017; Hylland et al., 2017b). In addition to organophosphates, AChE activity has also been shown to decrease after exposure to other contaminants such as certain compounds from aqueous oil water-soluble fractions (WAFs) from oil, some PAHs and trace metals may also inhibit AChE activity (Holth & Tollefsen, 2012; e.g. Olivares-Rubio & Espinosa-Aguirre, 2021; Roche et al., 2002; Vieira et al., 2009).

AChE activity in fish has been successfully applied in marine pollution monitoring with different fish species such as European flounder, sole, common dab, eelpout, European eel,

rainbow trout and red mullet (Asker et al., 2016; Burgeot et al., 2017; Evrard et al., 2010; Roubeix et al., 2023; Zinkl et al., 1991).

Bivalves: biomarkers

Metabolic disruption and immune disturbance

In marine bivalves, exposure to chemicals such as organophosphates, PCBs and trace metals may cause AChE inhibition (Escartín and Porte, 1997; Hamza-Chaffai et al., 1998; Galloway et al., 2002; Owen et al., 2002; Valbonesi et al., 2003; Bolton-Warberg et al., 2007). The observed response in bivalves implies metabolism impairment (Fulton & Key, 2001) related to altered tissue growth (Soreq and Seidman, 2001) and immune disturbance (Rickwood and Galloway, 2004).

Accordingly, AChE inhibition in bivalves has been recommended as a sensitive biomarker for marine pollution monitoring (Cajaraville et al., 2000) and has been measured in gills and foot of various species (oysters, clams and mussels) in field studies and monitoring programmes (Bodin et al., 2004; Bocquené et al., 2004; Kuprijanov et al., 2024; Lastumäki et al 2020; Lehtonen et al., 2003, 2016; Matozzo et al, 2005; Turja et al 2020, 2023).

Genotoxicity - micronucleus frequency

The mechanism for MN is the same in bivalves as for fish. The MN assay has been used in a range of different bivalve species, including e.g. *Mytilus edulis, Mytilus trossulus, Macoma balthica* and *Chlamys islandica.* The newly updated ICES TIMES protocol (no 66) includes detection of abnormal chromosome numbers as well as chromosome breakage (clastogenicity). The authors stress the importance of defining background levels of MNs and other nuclear abnormalities that are used as biomarkers (Stankevičiūtė et al., 2022).

Membrane toxicity - lysosomal membrane stability in digestive gland

Lysosomes and their membranes are targets for chemical stressors in all eukaryotes and their disruption is a marker for health status (Moore et al., 2006). In mussels, lysosome membrane stability (LMS) in digestive gland and blood cells is a sensitive and well-established marker for impairment of the membrane integrity resulting from combined contaminant stress (Moore et al., 2004; Martínez-Gómez et al., 2015).

LMS in mussel digestive gland is determined using an enzyme cytochemical method upon frozen tissue sections, which is recommended for its ease of sampling and reproducibility (Moore et al., 2004). Digestive gland cells, which are the major site of intracellular digestion in mussels, are rich in lysosomes and constitute the main interface between the organism and its environment (Moore, 1990). This biomarker has previously been recommended for monitoring in OSPAR, UNEP/RAMOGE, HELCOM and UNEP/MAP (Davies and Vethaak, 2012). It has also been used as a biomarker in monitoring programmes carried out at regional

level or after oil spills (Kopecka et al., 2006; Viarengo et al., 2007; Brooks et al., 2011; Garmendia et al., 2011; Lekube et al., 2014).

Amphipods: developmental toxicity

Embryo aberration frequency in amphipods, as described by Sundelin and Eriksson (1998) reflects reproductive and developmental toxicity. While much of the research has focused on sediment-dwelling species, where aberrations reflect exposure to bioavailable contaminants such as persistent organic pollutants and heavy metals (e.g., Eriksson Wiklund et al., 2005; Ford et al., 2003; Kolesova et al., 2024), the method is applicable to all amphipod species, including littoral and nektonic species, from the Arctic Ocean (Bach et al., 2010; Camus & Olsen, 2008) to South European rivers (Lacaze et al., 2011).

Embryo aberrations may result from "primary" mechanisms, such as endocrine disruption (Jacobson et al. 2011), genotoxicity (Lacaze et al., 2011) or epigenetic modifications (Gorokhova et al. 2020) or "secondary" mechanisms, including oxidative stress (Löf et al. 2016a) and membrane disruption.

Embryo aberrations in amphipods are currently used to assess contaminant effects in HELCOM (HELCOM 2023b) and OSPAR countries (Tairova & Strand 2022, Tairova et al., 2024). Since 2011, embryo aberration frequency has been used as a supplementary indicator in HELCOM-guided holistic assessments for MSFD implementation, including HOLAS II (2018) and HOLAS III (HELCOM 2023a) State of the Baltic Sea Assessments. In these assessments, the influence of environmental factors, such as temperature and salinity has been established (Kolesova et al., 2024) and incorporated to derive basin-specific BAC values (HELCOM 2023b).

Gastropods: imposex/intersex

Imposex/intersex in marine gastropods is a highly specific and sensitive dose-dependent response to exposure to organotins, particularly the antifouling agent tributyltin (TBT) (Bryan et al., 1986). TBT was not initially considered/observed to be acutely toxic but instead caused extensive adverse effects on a longer time scale through the disruption of sexual development leading to masculinisation of female gastropods. The females develop male sex characteristics and have also been observed to become sterile or have impaired health (Oehlmann et al., 1996).

Imposex was included for assessment of biological effects within an environmental monitoring programme coordinated within OSPAR/ICES (Gubbins et al., Ch 25 in Davies and Vethaak, 2012; Oehlmann 2004) and is at this time the only mandatory biomarker in OSPAR and HELCOM monitoring programmes.

1.2 Bioassays

Bioassays can be divided into *in vivo* and *in vitro* bioassays. A distinction can also be made between broad-spectrum bioassays and bioassays based on a specific mechanism of action.

In *in vivo* bioassays as used in environmental monitoring, intact organisms are exposed to environmental matrices, i.e. water or sediment, or extracts or either (Table 2). The tests may be of short duration (lasting hours to days) and designed to identify acute effects, or of longer duration (days to months) to quantify chronic effects. They can be carried out in a laboratory or in the field (*in situ*).

Acute tests provide an initial screening and quantify effects such as mortality in a population of the test organism. They simulate a worst-case scenario. Chronic tests are designed to emulate the actual situation in nature. Endpoints include reduced reproduction or growth in the test organism. Chronic tests are generally more sensitive than acute tests, but they are also more expensive and require more complex exposure systems.

Cell-based *in vitro* bioassays are laboratory tests using cells or subcellular fractions isolated from organisms, cell-lines or modified bacteria. These tests are mechanism-based, of short duration (lasting from several minutes to several days), quick to perform, and small scale.

Table 2. Recommended bioassays. Brackets indicate that SGIMC suggested water and sediment bioassays, but not detailed methods.

Matrix	Organism	Species	J	S	W
Water	Sea urchin	*Paracentrotus*, *Echinus*, *Strongylocentrotus*		(X)	X
	bivalve embryo	*Crassostrea*, *Mytilus*, *Ostrea*		(X)	X
	crustaceans	*Tisbe*, *Nitocra*, *Acartia*, shrimps, mysids		(X)	X
	phytoplankton	*Skeletonema*, *Isochrysis*, *Phaeodactylum*			X
Sediment	macroalgae	*Ceramium tenuicorne**			
	bacteria	*Vibrio fischeri**	X	(X)	X
	sediment-dwelling	*Corophium* sp.	X	(X)	X
		Arenicola marina			

J: JAMP/preCEMP; S: SGIMC (OSPAR 2013); W: ICES WGBEC, * ISO protocols

Water assays

A range of species have been recommended for use in water bioassays (Table 2), both larval stages and adult. In addition to seawater samples, this range of assays may be used with sediment pore-water, elutriates or concentrated samples. A range of species are recommended for use in water bioassays (Table 2), with both larval and adult stages being tested. These bioassays can be applied not only to seawater samples but also to sediment pore water, elutriates, or concentrated samples, depending on the environmental context. Established guidelines exist for several widely used assays. ISO standards support other essential tests like the marine algal growth inhibition test and the *Vibrio fischeri* luminescent bacteria test.

Sediment bioassays

A limited range of organisms have been used in sediment toxicity assays (Table 2), and the endpoints that have been used are not very sensitive. In addition to European species, other amphipod species have also been used. A limited range of organisms that have been used in the past in sediment toxicity assays (Table 2) but more sensitive sediment tests have been developed expanding the use of target species (Lehtonen et al., 2018). These tests can be applied to various types of matrices, including whole sediment, sediment extracts, or elutriates. Commonly used test organisms include benthic invertebrates such as crustacean amphipods, annelids and larvae of bivalves and echinoderms. To improve the sensitivity of these tests, molecular markers are increasingly being utilised. Amphipods are often selected as test organisms in the sediment bioassay due to their intense sensitivity to the various pollutants. *Monoporeia affinis* has been used in sediment bioassays and field studies (Strode et. al, 2017, Dabrowska et.al, 2017, Berezina et. al, 2013). Other species, such as *Corophium volutator, Gmelinoides fasciatus, Pontogammarus robustoides*, and *Gammarus tigrinus*, can be used similarly. Additionally, the macroalgae *Ceramium tenuicorne* and the copepod *Nitocra spinipes* have proven effective for testing sediment toxicity. Alternative tests using laboratory cultivated species such as *Chironomus riparius, C. yoshimatsui, C. tentans, Lumbriculus variegatus* or *Hyallela azteca* has also been recommended in OECD and ISO guidelines.

2. Selection of species

It is not possible to monitor effects of contaminants in all marine organisms, therefore indicator species must be used. Ideally, a single species would be chosen to allow direct comparison spatially and temporally, but such a species does not exist. This guideline proposes to use species-specific assessment criteria to enable comparison between species. This means that, for each selected species, assessment criteria for relevant biomarkers must be established.

Available data indicate that it is not advisable to directly compare biological effects responses between species even when they belong to the same genus. For example, closely related mussel species and their hybrids within the genus *Mytilus* can easily be confused (see e.g. Brooks et al., 2015; Sussarellu et al., 2022). In such cases it is relevant to accurately determine the species at monitoring sites using methods such as DNA profiling.

2.1 Currently used indicator species for effect-based monitoring

The main species groups previously used in European monitoring programmes have been fish, bivalves, gastropods and amphipods. The list of current species used in European monitoring programmes highlights the diversity of species in ongoing programmes (Table 3). Historically, other species have also been used e.g. four-spot megrim in Spanish North Atlantic waters.

Table 3. Species currently in use in OSPAR and HELCOM monitoring programmes.

Fish species	**Country/Region**
flounder (*Platichthys flesus*)	FR, NL, PL, SE, UK (E/W), UK (Sc)
dab (*Limanda limanda*)	DE, FR, UK (E/W), UK (Sc)
Atlantic cod *(Gadus morhua*)	DE, NO, UK (Sc)
plaice (*Pleuronectes platessa*)	UK (Sc)
grey mullet (*Chelon labrosus*)	ES (Basque Country)
common sole (*Solea solea*)	FR
perch (*Perca fluviatilis*)	SE, FI, PL
eelpout (*Zoarces viviparus*)	SE, DK
herring (*Clupea harengus*)	FI, PL
Bivalve species	**Country/Region**
blue mussel (*Mytilus edulis*)	NO, FR, UK (Sc), DK
Mediterranean mussel (*Mytilus galloprovincialis*)	ES, FR
Pacific blue mussel (*Mytilus trossulus*)	Baltic countries
clam *(Macoma balthica)*	EE, LV
Gastropod species	**Country/Region**
dog whelk (*Nucella lapillus*)	DK, FR, IS, IE, NL, NO, PT, ES, UK (E), UK (Sc)
netted dog whelk (*Tritia reticulata*)	DK, NL, PT, ES
common periwinkle (*Littorina littorea)*	DK, NL
common whelk (*Buccinum undatum)*	DK
red whelk (*Neptunea antiqua)*	DK, FR
Ocenebra erinaceus	FR
Tritia nitida	ES, SE
mudsnail (*Peringia ulvae)*	PT, SE
Amphipods	**Country/Region**
Monoporeia affinis, *Pontoporeia femorata* *Gmelinoides fasciatus*, *Pontogammarus robustoides* *Gammarus tigrinus*, *Gammarus locusta* other *Gammarid* species	DK, EE, LV, SE

2.2 Specific considerations

Species to include in monitoring programmes should be representative of the monitoring area and chosen based on suitable and sensitive response to the selected effect indicators, abundance and geographic distribution and ease of sample collection.

Species to be chosen as indicator species in effect-based monitoring programmes should:

- be suitable for measuring selected effects and responses must be sensitive (as established in laboratory studies)
- be well established in the monitoring areas. Species with wide geographic distribution are more favourable as they can be more easily compared spatially, but this is not critical (use species-specific assessment criteria)
- be amenable for collection and sampling as well as the species and maintain sample quality (see chapter on design)
- have a well-established autecology (i.e. "know your monitoring species")

In addition to the above, assessment criteria for the relevant biomarkers must be established if they are not yet available.

It is important to maintain ongoing time series where suitable target species have previously been used, for time-trend analyses and refinement of assessment criteria.

2.3 Species-dependent contaminant exposure

Demersal, sediment-dwelling fish are a common target for monitoring biological effects of contaminants as they live in close contact with and feed near or in the sediment, where several inorganic and hydrophobic contaminants accumulate. This makes them likely to be exposed to these contaminants by different exposure routes.

Autecological characteristics of a species will influence exposure to and effects of such exposure, and it is not to be expected that different species in the same habitat will have identical exposure or responses. Relevant characteristics are e.g. whether the species is a filter-feeder (like bivalves used in monitoring) or detritivorous, planktonic feeder or a predator (fish).

The recommendation is to use species that have important ecological roles and ensure that responses are comparable across different species through using assessment criteria. There will be differences between taxonomic categories: responses in a fish should not be directly compared to a bivalve or a gastropod. This recommendation ensures that existing time-series can be continued but requires experimental studies for some species to determine assessment criteria.

2.4 Species declines

A consideration is future population stability of chosen species and therefore suitability for long-term monitoring. Population declines in species already used in existing monitoring programmes have been reported across Europe. Intertidal mussel beds, which have been

severely impacted by human activities in the past, are still decreasing in some countries (Norway, Spain, Sweden, Scotland), or have disappeared (from some sampling locations in France) (OSPAR, 2023). Research monitoring in the Basque country (Spain) is investigating use of oysters (*Magallana* sp.) as an alternative to monitoring in mussels.

Similarly, cod stocks in the North Sea have declined to historically low levels of abundance. Atlantic cod and mussels are widely used across Europe in existing monitoring programmes (Table 3). Population declines will impact those programmes and need to be considered when selecting monitoring species.

2.5 Recommended monitoring species

In line with previous guidelines and ongoing monitoring programmes in the North Atlantic and Baltic Sea, any of the species listed in Table 3 are considered suitable for monitoring. It is important that the monitoring species chosen is relevant for the area in question. It is also critical that assessment criteria are established for biomarkers in the selected species if they do not exist.

3. Assessment and quality assurance

3.1 Assessment

We need to be able to quantify and compare contaminant-related effects in different geographical regions. The optimal situation would have been to use organisms that are found throughout Europe, as was the hope for the "mussel watch" concept in the 1970-80s. It has become increasingly clear that this is not feasible, mainly because of the limited distribution of even the most common species, but also because we now know that even related species may respond differently to contaminant exposure (Sussarellu et al., 2022).

The solution to this issue is to target ecologically relevant species in each region, or even where necessary sub-region, and use assessment criteria for each biomarker in selected species. Earlier Europe-wide assessment using five different species has demonstrated that this is feasible (Hylland et al., 2017a, Vethaak et al., 2017).

In the earlier guidelines, two assessment criteria were developed: a background assessment criterion (BAC), below which responses are at a level found in unpolluted habitats, and an environmental assessment criterion (EAC), above which deleterious damage to one or more species in the area monitored is expected (SGIMC, 2011).

Establishing a BAC requires data collection: it is determined by collecting the target species in more or less pristine areas, followed by analyses of the relevant biological effect methods. BAC can then be calculated (SGIMC, 2011). The appropriateness of the metric will depend on the expected variability of each biomarker in each species. In previous guidelines, a 90th percentile which has been used for biomarkers. With increasing data availability for different species from pristine areas, the BACs can be more precisely adjusted at the biomarker-species level. Further work is required if it is deemed necessary to determine area-specific BACs.

It is currently recommended to only use BAC and not EAC. This is due to the uncertainty of the development of EAC values. Further work is needed to develop EACs and some strategies are under consideration. The current recommendation will mean that each biomarker will generate either “good status” (below BAC) or “polluted” (above BAC) and the quality of the BAC is crucial. It also means that there is a need to include a set of biomarkers for a satisfactory assessment. An approach combining results from different analyses has been developed through the CHASE project in HELCOM (Annex B (HASEC 25/05/04Add3)).

As stated above, the proposed approach makes it possible to compare responses in species across regions and sub-regions, although it is preferable to use one species within a single monitoring campaign.

As will be evident from tables 4 and 5 there are assessment criteria for most recommended biomarkers and many of the relevant species, as well as for bioassays.

Table 4. Currently available general BACs for biomarkers.

Biomarker	Trophic level	Species
Neoplasia or tumors	flatfish	dab
Vitellogenin in plasma	fish	cod, flounder
Micronucleus frequency in erythrocytes	fish	flounder, dab, eelpout, cod, red mullet
DNA strand breaks in erythrocytes	fish	cod, dab
Cytochrome P4501A activity (EROD) in liver	fish	cod, dab, flounder, plaice, red mullet, eelpout, perch
AChE activity in muscle	fish	dab, flounder, red mullet
Lysosomal membrane stability (LMS) in digestive gland	bivalves	all relevant species
Micronucleus frequency in haemocytes	bivalves	*Mytilus* sp., *Macoma balthica*
AChE activity in gill or foot	bivalves	*Mytilus* sp., *Macoma balthica*
Embryo malformation	amphipods	*Monoporeia affinis, Pontoporeia femorata, Gammarus* spp*., Gmelinoides fasciatus, Pontogammarus robustoides, Gammarus tigrinus, Corophium volutator*
Imposex / Intersex	gastropods	*Nucella lapillus*, *Tritia reticulata, Buccinum undatum, Neptunea antiqua, Peringia ulvae, Littorina littorea*

Assessment criteria are available for all recommended bioassays (Table 5). Whole-organism assays are important due to their ecological relevance, but the protocols currently available do not appear to be sufficiently sensitive to produce data that will protect organisms in the relevant habitats (pelagic, sediment).

Table 5. Bioassays for which assessment criteria are available.

Matrix	Organism	Species	Endpoints
Water	sea urchin larva	*Paracentrotus sp Echinus sp*, *Strongylocentrotus sp*	% abnormality % growth
	bivalve embryo	*Crassostrea sp*, *Mytilus sp*, *Ostrea sp*	% abnormality
	crustaceans	*Tisbe sp*, *Nitocra sp*, *Acartia sp*, shrimps, mysids	mortality
	phytoplankton	*Skeletonema sp*, *Isochrysis sp*, *Phaeodactylum sp*	primary production
Sediment	sediment-dwelling species	*Corophium* sp, *Arenicola marina*	mortality

3.2 Quality Assurance

Quality assurance (QA) in biological effects monitoring is essential to ensure collected data are of the highest standard and comparable with other laboratories during large regional assessments. Monitoring countries that submit their biological effects data to ICES are expected to participate in appropriate QA programmes incorporating proficiency testing for the selected methods. Participation in these programmes, specifically their corresponding statement of performance, should be documented and included within the master submission of monitoring data to ICES. These data may be omitted from regional assessments in the absence of participation QA.

The Biological Effects Quality Assurance in Monitoring (BEQUALM) programme was an EU funded research programme initiated in 1998. BEQUALM was designed to provide quality standards for a wide range of biological effects techniques used in marine monitoring and to provide a framework for monitoring compliance of European laboratories that generate biological effects data within national and international monitoring programmes. BEQUALM now operates as a self-funded QA programme for biological effects techniques.

BEQUALM proficiency testing over the last 5 years (2020-2024) has included micronuclei formation in mussel haemocytes, polycyclic aromatic hydrocarbons (PAH) metabolites in fish bile, and ethoxyresorufin *O*-deethylase (EROD) activity in fish liver. Results can be found at the BEQUALM website. BEQUALM is currently including the incorporation of

acetylcholinesterase (AChE) activity in fish muscle, and vitellogenin in fish blood plasma into the ‘Biomarker’ component of the programme. This will include optimisation of how the inclusion of these additional methods are best conducted during future years. Additional planned BEQUALM activities in 2025 include EROD activity in fish liver, PAH metabolites in fish bile, and micronuclei formation in fish erythrocytes. These above activities will be facilitated by samples generated by the EU Horizon 2023 project CONTRAST (www.contrastproject.eu). Furthermore, BEQUALM will implement a liver histopathology proficiency test for the detection of hepatic neoplasms under the ‘Whole Organism’ component. This will be achieved using samples obtained during the UKs Clean Seas Environmental Monitoring Programme (CSEMP).

Moving forward, it is anticipated that proficiency tests will be organised for each test method within a regular interval, co-ordinated through ICES WGBEC and BEQUALM. A new BEQUALM website will be available shortly (www.bequalm.org) and will reflect the renewed future direction of BEQUALM. It will contain updated information on upcoming activities as well as a report archive of previous proficiency tests.

4. Monitoring design

The design of effect-based monitoring programmes comprises all activities from selecting the location, collecting samples to activities from characterisation of the collection site to analysis in the laboratory and treatment of data.

Although some parts of the design will need to be coordinated for different methods, such as selection of site, other recommendations may differ for e.g. bioassays and biomarkers.

Monitoring design comprises:

(i) deciding location(s)/area(s)
(ii) characterisation of collection site and exposure assessment
(iii) selecting individuals
(iv) collection method
(v) number of the selected organism(s) of appropriate size and/or sex and age

Guidelines for sampling, transport and storage of water and/or sediment for subsequent testing or extraction is available elsewhere (i.e. relevant ICES TIMES protocols for individual methods). A brief background for the methods presented can be found in section 1 and in more depth in Davies and Vethaak (2012).

Earlier OSPAR guidelines separated between general monitoring and contaminant-specific monitoring. Both were directed towards detecting and quantifying effects of contaminants. Following on from the framework developed under SGIMC (Davies and Vethaak, 2012), the current guideline recommends including a combination of methods to cover the most important mechanisms of toxicity at selected locations (Hylland et al., 2021). Including different mechanisms of toxicity will ensure a holistic assessment of contaminant risks. In practice, there should be no difference in the design between locations in terms of the programme implemented, whether they are perceived to be polluted or pristine.

The concentration of chemicals in tissues is useful for modeling food chain transfer and bioaccumulation in organisms within a specific habitat. Measured tissue concentrations can provide important information about chemical exposure, particularly for substances that persist in biological systems. In species intended for human consumption, tissue residues are a critical parameter for food safety assessments and regulatory compliance.
However, tissue concentrations alone cannot predict potential toxic effects in the organism (e.g., Altenburger et al., 2019; Brack et al., 2017; Hylland et al., 2021; Vethaak et al., 2017). Many contaminants, including both known and unknown chemicals of concern, can induce biological effects without significant accumulation. This occurs when substances are rapidly metabolized, are not easily extractable for detection, or exert effects at concentrations too low to be identified by standard chemical analyses.

Therefore, monitoring programs for contaminants in marine ecosystems must integrate both chemical analyses (to quantify exposure and accumulation) and effect-based analyses (to

assess biological responses) using suitable matrices. This combined approach ensures a more accurate characterization of environmental contamination and its potential impacts. The programme described here can be used stand-alone or in spatial or temporal monitoring since results from different biomarkers can be summed up to provide an integrated assessment (Hylland et al., 2017a).

4.1 Locations/areas and season for monitoring

The effects of contaminants on selected organisms will vary based on their exposure and the species being monitored. It is therefore important to ensure there is sufficient information available to determine potential exposure, considering the time taken to detect a response (e.g. a biomarker). This will entail assessing movement for mobile species and movement of water for all, taking into account depth and hydrography.

Prior to selecting a sampling location, the area represented by the selected species should be assessed. For fish this will depend on the species and knowledge of seasonality and migration behaviour (e.g. through FishBase). Most of the recommended methods have short response times (i.e. days or weeks), and for demersal species it is generally been assumed that they reflect the area in which they are collected. The extent of the area must however be estimated, using both general and local knowledge of the species in question. In the case of demersal species, previous knowledge concerning geographical location of the main fishing grounds for the species should also be considered, since it will provide certain advantages in relation to the cost/benefit in the sampling design.

Sampling must generally be performed outside the reproductive period for the species in question. For most fish species this will mean the spring, but it can be more challenging for bivalves that may spawn over a prolonged period. In case that is not possible to perform the sampling outside the spawning season, the use of juveniles with a low gonadosomatic index should be considered. The collection of female amphipods for embryo malformation analysis is an exception to this general recommendation. Specifically, gravid females should be collected when the embryos are in late developmental stages (e.g. mid to late January in the Baltic Sea).

4.2 Exposure assessment

The recommended species for monitoring are fish species, bivalves, amphipods and gastropods (Section 2). A range of fish species are currently in use and will continue to be used in the future in marine monitoring programs in European countries.

Exposure to contaminants will be through water, sediment and/or diet, depending on both species and the relevant contaminant. Tissue concentrations of contaminants in the target species is a preferred measure of exposure for bioaccumulating substances. Polycyclic aromatic hydrocarbon (PAH) metabolites in fish bile are a special case, since it is a more

precise estimate for PAH exposure than tissue concentrations, and will reflect recent exposure, i.e. days and weeks.

Some contaminants will not bioaccumulate in fish and exposure assessment may then either rely on concentrations in other organisms in the same habitat such as filter-feeding bivalves, or water or sediment, depending on the properties of the substance. For some contaminants, exposure may be most precisely assessed using data for biomarkers measured in the target species itself, such as vitellogenin for estrogenic chemicals or AChE inhibition in fish for organophosphate or carbamate exposure (cf section 1).

Description of analytical methods for contaminants is not within the scope of this guideline, with one exception: analyses for PAH metabolites in fish bile. PAHs are widely present in aquatic ecosystems and are a cause of concern as they have been linked to tumours and other adverse effects in exposed organisms. While PAHs can bioaccumulate in invertebrates, they are generally metabolised and excreted in fish and other vertebrates. Metabolites can be quantified in bile or urine, providing an indicator of recent PAH exposure (Kammann et al., 2013). Analysis of PAH metabolites in bile is a sensitive marker of recent PAH exposure, at a scale of days up to a few weeks, after which the PAHs will have been excreted (Richardson et al., 2004). There are three main types of analyses: direct analysis using the fluorescent properties of PAHs, deconjugation, then separation by either liquid chromatography (LC) or gas chromatography (GC), followed by detection and quantification by mass spectrometry (MS) or fluorescence. The two chromatographic methods have different scopes, LC mainly used to separate metabolites with more than four benzene rings (i.e. pyrene upwards), whereas GC is preferred for smaller PAHs. All three have been used in the past, and there are appropriate assessment criteria for a range of relevant species.

For filter-feeding bivalves such as mussels or sediment-dwelling filter-feeding mussels, exposure is through water and particles in water. Dispersion models may be used to estimate the area represented by the selected site for mussels and sediment concentrations for sediment-dwelling bivalves (assuming substances in water will associate with particles and end up on the sediment surface). Stratification in the period prior to sampling also needs to be considered. Rocky shore gastropods will be exposed through water and diet.

4.3 Collection method

A sufficient number of the target organism must be collected while avoiding levels of stress that could affect effect responses. For fish, appropriate methods are gill nets, fyke nets or trawling. Rocky shore organisms are collected by hand, sediment-dwelling bivalves by grab or sledge.

To carry out biomarker analyses in fish, it is necessary that blood samples be obtained while the animal is still alive and that liver, muscle and brain samples be obtained immediately after

the sacrifice of the animal. These requirements imply, therefore, handling of the specimens so that they remain alive after their capture.

Organisms should preferably be sampled immediately following collection. Storage in pens, tanks or cages prior to sampling increases the risk of sampling artefacts or exposure to contaminants present in that location and need to be carefully evaluated.

Keeping live fish after trawling (where a significant number of specimens are usually captured) and before euthanasia can be convenient to keep them alive until processing. This could be done on the deck of the boat, keeping them in a tank with open seawater circulating from the capture area until processing (10-30 minutes). It is recommended to cover the top of the tank to minimise stress. All procedures need to comply with ethical guidelines in each country and follow consultation with appropriate authority.

Organisms collected in one area may be contaminated or decontaminated during storage at another location, or organisms may be stressed due to change in temperature and salinity during storage. Time in storage as well as storage conditions needs to be documented. Whichever species is collected, it is clearly essential that individuals are kept under conditions that ensure that they are unstressed prior to sampling and during transport. This may entail using e.g. cooling in tanks or thermoinsulated containers.

4.4 Caging

Caging involves transplantation of bioindicator organisms. Through caging it is possible to ensure exposure of organisms at sites along a pollution gradient or at hot spots. Mussels have been widely used for caging due to exposure both through water and waterborne particles (e.g. Lehtonen et al., 2019; Marigómez et al., 2013; Regoli et al., 2004), but also other species, e.g. fish, have been used (Hylland et al., 2010). The potential use of passive samplers of chemicals in the caging system is an advantage.

Among the main benefits of using the caging method is that bioaccumulation dynamics of contaminants and their biological effects can be assessed in parallel in temporal and spatial scales. Observations can be carried out along suspected pollution gradients or hot-spot sites without the uncertainties related to potential long-term physiological/genetic adaptation of populations inhabiting chronically polluted sites or the common practical problem of the lack of suitable indicator organisms in the areas of concern. Also, the approach effectively tackles problems related to the variability in environmental factors since the caging sites and the duration of the experiment can be carefully selected according to available *a priori* information. On the other hand, exposure of organisms in cages is not the same as organisms in nature and they may starve during deployment (particularly relevant for non-filter-feeding organisms such as fish).

4.5 Numbers, sampling and sample treatment

The number sampled will depend on the known variability of the biomarker and the increase or decrease in the parameter viewed as biologically significant. The latter is already included in the background assessment guideline (BAC). Numbers for each biomarker are provided in the appropriate protocol and differ for fish and invertebrates (see list of TIMES protocols in Annex C (HASEC 25/05/04Add4)).

The design of monitoring programmes with different species and many stations needs to take into account both the welfare of organisms and to select strategies to ensure samples of high quality.

5. Effect-based methods (EBM) and effect-directed analysis (EDA)

Biomarkers and bioassays evaluate the potential toxicity of contaminants in intact organisms (biomarker) or field-collected water or sediment (bioassay). Another option is to extract contaminants from environmental matrices, in this context sediment or water, for subsequent testing with cell-based methods. The toxicity of such extracts may also be tested using bioassay organisms (see section 1).

Direct testing of elutriates or extracts is referred to as effect-based methods (EBM), whereas sequential fractionation and testing of fractions is referred to as Effect-Directed Analysis (EDA) (Klamer et al., Ch 23 in Davies & Vethaak, 2012).

EDA has the potential to contribute to understanding the effects of chemical pollutants at different levels of biological organization, while providing helpful information for the prioritization and regulation of emerging contaminants present in the aquatic environment.

Several EDA tools and protocols are available (Brack et al., 2016; Klamer et al., Ch 23 in Davies & Vethaak, 2012), including sample preparation (sieving, drying, homogenizing), sample extraction, extract concentration and clean-up (manual or automatic). ISO guidelines and OECD technical guidelines are available for testing EDA extracts in some *in vitro* assays.

The advantages of EBM or EDA compared to direct measurement of effects in water or sediment is that extraction removes a range of potentially confounding factors such as salinity, temperature, grain size and organic content. For polar (water soluble) extracts, there may be further issues with e.g. ammonia in sediments. Such extracts can also be concentrated or further fractionated to aid in identifying the main toxic substances in the sample.

An important drawback with EBM and EDA is that the extracts will not represent true exposure in the ecosystem, so the environmental relevance is dubious. Recovery of different chemicals from extraction columns introduces a substantial uncertainty in toxicity assessment. Extraction from sediment may change the toxicity of some toxicants, e.g. metals, by changing the pH or redox conditions. The extraction of weak acids and bases may also be affected by changes in pH. The composition of final extracts will in most cases not reflect the situation organisms in sediment would be exposed to.

5.1 Extraction and fractionation

Extraction methods have traditionally been developed for chemical analyses and have therefore not been optimised for bioassays, which may result in a misrepresentation of the actual toxicity in environmental matrices. Different extraction techniques for water and wastewater samples has shown that sample preparation and extraction critically affect the outcome of bioassays when assessing the toxicity of water samples (Abbas et al., 2019).

A protocol for the extraction of water and sediment for subsequent *in vitro* analyses, relevant for both EBM and EDA, is described in Klamer et al. (Ch 23, in Davies & Vethaak, 2012). A general protocol for fractionation of extracts can be found in Brack et al. (2016). This is however a critical issue when considering the use of extracts for toxicity determination in a monitoring context.

5.2 EBM and EDA effect methods

EBM involves testing an environmental extract without subsequent fractionation, which generally implies using cells or organisms with endpoints that represent a wide range of responses. The same methods are generally used for fractions derived in EDA.

Any bioassay method (*in vivo* or *in vitro*) used in monitoring (cf section 1) can be applied in EBM or EDA. In addition, cell-based methods may be used (see below). To comply with EBM and EDA requirements, assays need to be robust, small-volume and high-throughput. For practical reasons, short-term assays are preferable. The most suitable assays for EDA should fulfil the following requirements (Brack et al., 2016): high throughput, allowing a large number of samples and fractions to be tested, low sample volume, allowing a wide range of test concentrations to be tested, high sensitivity, to detect the effects of environmental toxicants at low concentrations, short test turnaround, high test specificity, high availability/accessibility and, finally, cost effectiveness.

In vitro assays that indicate alterations in metabolism, or molecular events that may initiate responses at higher levels of organisation, as well as adaptive stress responses, are typically suitable for EBM or EDA. Also useful are *in vitro* tests that, despite not anticipating effects on organisms/populations, can serve to identify suspect toxicants. *In vitro* tests that address specific modes of action (MoA) are preferable because they may serve to identify specific contaminants that may have effects at low concentrations. MoA-specific *in vitro* assays suitable for environmental monitoring are available for e.g. receptor-mediated endocrine effects, genotoxicity and mutagenicity, activation of metabolism, adaptive stress responses, photosynthesis inhibition and cell line-specific cytotoxic effects. In addition to more mechanism-specific effects, whole organism in vitro tests can also be an important component to identify specific mechanisms of action, e.g. algal tests for herbicides or larval tests for developmental toxicants or neurotoxicants.

Different receptor and cell-based bioassays, using fish, rat and human cell models, have previously been successfully used to estimate the biological activity of sediment-bound pollutants, integrating their interactions and covering endpoints such as acute and long-term toxicity, oxidative-stress and endocrine disrupting effects (Creusot et al., 2010; Schnell et al., 2013; Fernandes et al., 2014; Pérez-Albaladejo et al., 2016; Blanco et al., 2018).

For testing marine sediments, fish cell-based bioassays such as oxidative stress, EROD activity and cell viability have been also complemented with luciferase-based reporter gene assays involving contaminant-activated receptors (Thomas et al., 2004; Klamer et al., 2005; Vethaak et al., 2017, Martínez-Gómez et al., 2020; Goksøyr et al., 2021). The use of primary cell cultures from invertebrates to investigate the impact of pollutants in the environment have recently been described (García-Velasco et al., 2023).

A single bioassay cannot cover all responses that may be induced by complex chemical mixtures, usually a range of tests covering different MoAs is required, e.g. cytotoxicity, biotransformation, endocrine disruption, genotoxicity and inhibition of enzyme activity.

5.3 EBM and EDA - conclusion

EBM and EDA are not considered appropriate for regular monitoring at this time. In follow-up studies, both EBM and EDA can contribute to clarify apparent incoherence between the presence of high levels of pollutants in both water and sediment and a lack of response in biomarkers. EBM can potentially provide complementary information about mixture effects and EDA can contribute to identifying mechanisms of toxicity and responsible contaminants that are not currently monitored.

Combined use of pore-water extraction and *in vitro* techniques in sediment monitoring can contribute to identifying potentially toxic contaminants, taking into consideration that biomarkers for sediment toxicity are limited.

In summary, *in vitro* tests may provide information about the specific mode of action of environmental contaminants, but there will always be a discussion about the environmental relevance of extracts.

6. National activities

Sixteen European countries have on-going effect-based monitoring programmes in the East Atlantic and Baltic Sea: Denmark, Estonia, Finland, France, Germany, Iceland, Ireland, Italy, Latvia, The Netherlands, Norway, Poland, Portugal, Spain, Sweden, UK (England-Wales) and UK (Scotland) (Table 1). Monitoring programmes before 2020 have not been included.

Taxonomic groups

Only one country is monitoring biological effects using all four taxonomic groups recommended by SGEFF, although for some taxonomic groups the methods are different than those recommended by SGEFF.

Fish are the most popular taxonomic group monitored for biological effects (12 countries), followed by gastropods (9 countries), bivalves (8 countries) and amphipods (3 countries).

Fish monitoring

Fish monitoring using biological effects is performed annually in eight countries. In one country a different sampling frequency is used (1, 3 or 6 years), depending on the site-specific risk(s) for selected hazardous substances. For the remaining countries, sampling frequency is every 2, 3 and 4 years.

More than half of the countries (10 countries) monitor two or more biomarkers in fish recommended by SGEFF, the most popular is bile PAHs metabolites (7 countries), followed by EROD activity, micronuclei, AChE activity, neoplasia or tumours in liver and DNA strand breaks. One country currently monitors vitellogenin in fish plasma.

Bivalve monitoring

Eight countries have ongoing effect-based monitoring programmes using mussels and 5 of the countries use methods recommended by SGEFF. Bivalve target species in on-going monitoring programmes were *Mytilus* sp. and *Macoma balthica.* Bivalve monitoring is generally performed annually, the only exception is one country where the sampling frequency is every three years (as a part of a rotating sampling plan of the coast)

Monitoring of imposex/intersex in gastropod molluscs

Monitoring of imposex/intersex in gastropod molluscs is currently performed by 9 countries. Sampling frequency of imposex differs among countries and range from yearly frequency (in

3 countries), every 3-4 years (in 4 countries), every 5 years (in one country) and every 6 years (in one country).

Gastropod target species in ongoing monitoring programmes are *Nucella lapillus, Littorina littorea, Buccinum undatum, Neptunea antiqua, Ocenebra erinaceus, Tritia reticulata, Tritia nitida, Peringia ulvae*.

Embryo malformation monitoring in amphipods

Three countries monitor embryo malformation in amphipods. These three countries are Baltic countries and use the same target species *Monoporeira affinis,* with the addition of a second target species *Pontoporeira femorata* in one of these countries.

Table 6. Biomarkers included in current national monitoring programs. Biomarkers recommended by SGEFF are in bold.

Organism	Biomarker	DK	EE*	FI	FR	GE	IC	IR	LV	NL	NO	SP	SE	PL	PT	UK-E	UK-Sc
Fish	**Neoplasia in liver**				X	X					X					X	X
	VTG												X				
	PAH metabolites	X			X	X				X	X					X	X
	EROD	X									X		X			X	X
	Micronuclei				X									X		X	X
	AChE				X						X		X			X	
	LMS-Cyt			X	X												
	DNA strand breaks				X						X		X				
	Fish diseases					X				X	X					X	X
	Intersex				X												
	Malformed brood	X															
	Reproductive success	X															
	CAT, GR, GST												X				
	CYP1A										X						
	% Blood cells												X				
	DNA adducts										X						
Bivalves	**AChE**		X		X				X			X					
	MN				X							X					
	LMS-Cyt				X						X						
	LMS-NRR	X											X				
	Metallothionein																
	SoS										X						X
	COMET assay				X												
	Histopathology								X								
	CAT, GR, GST		X						X								
Gastropods	**Imposex/intersex**	X			X		X	X		X		X	X		X	X	X
Amphipods	**Malformed embryos**		X						X				X				

*used in long-term monitoring and assessments, but not part of official national programme

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