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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# Executive summary

The Study Group on developing new guidelines for the monitoring of biological EFFects of contaminants (SGEFF) was established by OSPAR and HELCOM in 2022 for a 2-year period. The group included 22 experts from both regions, ensuring that regional differences were considered. The final SGEFF report (2024) recommended 11 biological-effect methods to cover the broad range of toxicological responses, as well as relevant species groups (Table 1). These methods have well-documented ICES TIMES protocols and are included in intercalibration and QA programmes.

# 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 90$^{th}$ 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<br>% 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

## References


Abbas, A., Schneider, I., Bollmann, A., Funke, J., Oehlmann, J., Prasse, C., Schulte-Oehlmann, U., Seitz, W., Ternes, T., Weber, M., Wesely, H., & Wagner, M. (2019). What you extract is what you see: Optimising the preparation of water and wastewater samples for in vitro bioassays. *Water Research*, *152*, 47–60. https://doi.org/10.1016/j.watres.2018.12.049

Al-Sabti, K., & Metcalfe, C. (1995). Fish micronuclei for assessing genotoxicity in water. *Mutation Research*, *343*(2), 121–135. https://doi.org/10.1016/0165-1218(95)90078-0

Altenburger, R., Brack, W., Burgess, R. M., Busch, W., Escher, B. I., Focks, A., Mark Hewitt, L., Jacobsen, B. N., de Alda, M. L., Ait-Aissa, S., Backhaus, T., Ginebreda, A., Hilscherová, K., Hollender, J., Hollert, H., Neale, P. A., Schulze, T., Schymanski, E. L., Teodorovic, I., … Krauss, M. (2019). Future water quality monitoring: improving the balance between exposure and toxicity assessments of real-world pollutant mixtures. *Environmental Sciences Europe*, *31*(1). https://doi.org/10.1186/s12302-019-0193-1

Asker, N., Albertsson, E., Wijkmark, E., Bergek, S., Parkkonen, J., Kammann, U., Holmqvist, I., Kristiansson, E., Strand, J., Gercken, J., & Förlin, L. (2016). Biomarker responses in eelpouts from four coastal areas in Sweden, Denmark and Germany. *Marine Environmental Research*, *120*, 32–43. https://doi.org/10.1016/j.marenvres.2016.07.002

Augusti-Tocco, G., Biagioni, S., & Tata, A. M. (2006). Acetylcholine and regulation of gene expression in developing systems. *Journal of Molecular Neuroscience: MN*, *30*(1-2), 45–48. https://doi.org/10.1385/JMN:30:1:45

Bach, L., Fischer, A., & Strand, J. (2010). Local anthropogenic contamination affects the fecundity and reproductive success of an Arctic amphipod. *Marine Ecology Progress Series*, *419*, 121–128. https://doi.org/10.3354/meps08872

Barsiene, J., Lazutka, J., Syvokiene, J., Dedonyte, V., Rybakovas, A., Bagdonas, E., Bjornstad, A., & Andersen, O. K. (2004). Analysis of micronuclei in blue mussels and fish from the Baltic and North Seas. *Environmental Toxicology*, *19*(4), 365–371. https://doi.org/10.1002/tox.20031

Baynes, A., Lange, A., Beresford, N., Bryden, E., Whitlock, K., Tyler, C. R., & Jobling, S. (2023). Endocrine Disruption Is Reduced but Still Widespread in Wild Roach (*Rutilus rutilus*) Living in English Rivers. *Environmental Science & Technology*, *57*(34), 12632–12641. https://doi.org/10.1021/acs.est.3c02854

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., Ausili, A., & Regoli, F. (2012). A multidisciplinary weight of evidence approach for classifying polluted sediments: Integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environment International*, *38*(1), 17–28. https://doi.org/10.1016/j.envint.2011.08.003

Berezina, N. A., Strode, E., Lehtonen, K. K., Balode, M., & Golubkov, S. M. (2013). Sediment quality assessment using *Gmelinoides fasciatus* and *Monoporeia affinis* (Amphipoda, Gammaridea) in the northeastern Baltic Sea. *Crustaceana*, *86*(7-8), 780–801. https://doi.org/10.1163/15685403-00003215

Blanco, M., Pérez-Albaladejo, E., Piña, B., Kušpilić, G., Milun, V., Lille-Langøy, R., Karlsen, O. A., Goksøyr, A., & Porte, C. (2018). Assessing the environmental quality of sediments from Split coastal area (Croatia) with a battery of cell-based bioassays. *The Science of the Total Environment*, *624*, 1640–1648. https://doi.org/10.1016/j.scitotenv.2017.10.055

Bocquené, G., Chantereau, S., Clérendeau, C., Beausir, E., Ménard, D., Raffin, B., Minier, C., Burgeot, T., Leszkowicz, A. P., & Narbonne, J.-F. (2004). Biological effects of the “Erika” oil spill on the common mussel (*Mytilus edulis*). *Aquatic Living Resources*, *17*(3), 309–316. https://doi.org/10.1051/alr:2004033

Bocquené, G., & Galgani, F. (1998). Biological effects of contaminants: cholinesterase inhibition by organophosphate and carbamate compounds. *ICES Techniques in Marine Environmental Sciences*, *22*, 12. https://doi.org/10.17895/ices.pub.5048

Bodin, N., Burgeot, T., Stanisière, J. Y., Bocquené, G., Menard, D., Minier, C., Boutet, I., Amat, A., Cherel, Y., & 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. Toxicology & Pharmacology:*

*CBP*, *138*(4), 411–427. https://doi.org/10.1016/j.cca.2004.04.009

Bolton-Warberg, M., Coen, L. D., & Weinstein, J. E. (2007). Acute toxicity and acetylcholinesterase inhibition in grass shrimp (*Palaemonetes pugio*) and oysters (*Crassostrea virginica*) exposed to the organophosphate dichlorvos: laboratory and field studies. *Archives of Environmental Contamination and Toxicology*, *52*(2), 207–216. https://doi.org/10.1007/s00244-005-0325-z

Brack, W., Ait-Aissa, S., Burgess, R. M., Busch, W., Creusot, N., Di Paolo, C., Escher, B. I., Hewitt, L. M., Hilscherova, K., Hollender, J., & Others. (2016). Effect-directed analysis supporting monitoring of aquatic environments—an in-depth overview. *The Science of the Total Environment*, *544*, 1073–1118. https://doi.org/10.1016/j.scitotenv.2015.11.102

Brack, W., Dulio, V., Ågerstrand, M., Allan, I., Altenburger, R., Brinkmann, M., Bunke, D., Burgess, R. M., Cousins, I., Escher, B. I., Hernández, F. J., Hewitt, L. M., Hilscherová, K., Hollender, J., Hollert, H., Kase, R., Klauer, B., Lindim, C., Herráez, D. L., … Vrana, B. (2017). Towards the review of the European Union Water Framework Directive: Recommendations for more efficient assessment and management of chemical contamination in European surface water resources. *The Science of the Total Environment*, *576*, 720–737. https://doi.org/10.1016/j.scitotenv.2016.10.104

Brooks, S., Harman, C., Zaldibar, B., Izagirre, U., Glette, T., & Marigómez, I. (2011). Integrated biomarker assessment of the effects exerted by treated produced water from an onshore natural gas processing plant in the North Sea on the mussel *Mytilus edulis*. *Marine Pollution Bulletin*, *62*(2), 327–339. https://doi.org/10.1016/j.marpolbul.2010.10.007

Brooks, S. J., Farmen, E., Heier, L. S., Blanco-Rayón, E., & Izagirre, U. (2015). Differences in copper bioaccumulation and biological responses in three Mytilus species. *Aquatic Toxicology (Amsterdam, Netherlands)*, *160*, 1–12. https://doi.org/10.1016/j.aquatox.2014.12.018

Bryan, G. W., Gibbs, P. E., Hummerstone, L. G., & Burt, G. R. (1986). The decline of the gastropod *Nucella lapillus* around south-west England: Evidence for the effect of tributyltin from antifouling paints. *Journal of the Marine Biological Association of the United Kingdom. Marine Biological Association of the United Kingdom*, *66*(3), 611–640. https://doi.org/10.1017/s0025315400042247

Burgeot, T., Akcha, F., Ménard, D., Robinson, C., Loizeau, V., Brach-Papa, C., Martínez-Gòmez, C., Le Goff, J., Budzinski, H., Le Menach, K., Cachot, J., Minier, C., Broeg, K., & Hylland, K. (2017). Integrated monitoring of chemicals and their effects on four sentinel species, Limanda limanda, Platichthys flesus, Nucella lapillus and Mytilus sp., in Seine Bay: A key step towards applying biological effects to monitoring. *Marine Environmental Research*, *124*, 92–105. https://doi.org/10.1016/j.marenvres.2016.10.009

Cajaraville, M. P., Bebianno, M. J., Blasco, J., Porte, C., Sarasquete, C., & Viarengo, A. (2000). The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. *The Science of the Total Environment*, *247*(2-3), 295–311. https://doi.org/10.1016/s0048-9697(99)00499-4

Cajaraville, M. P., Olabarrieta, I., & Marigomez, I. (1996). In vitro activities in mussel hemocytes as biomarkers of environmental quality: a case study in the Abra Estuary (Biscay Bay). *Ecotoxicology and Environmental Safety*, *35*(3), 253–260. https://doi.org/10.1006/eesa.1996.0108

Camus, L., & Olsen, G. H. (2008). Embryo aberrations in sea ice amphipod *Gammarus wilkitzkii* exposed to water soluble fraction of oil. *Marine Environmental Research*, *66*(1), 221–222. https://doi.org/10.1016/j.marenvres.2008.02.074

CORESET. (2013). HELCOM core indicators: Final report HELCOM CORESET project. *Balt. Sea Environ. Proc*, *136*.

Creusot, N., Kinani, S., Balaguer, P., Tapie, N., LeMenach, K., Maillot-Maréchal, E., Porcher, J.-M., Budzinski, H., & Aït-Aïssa, S. (2010). Evaluation of an hPXR reporter gene assay for the detection of aquatic emerging pollutants: screening of chemicals and application to water samples. *Analytical and Bioanalytical Chemistry*, *396*(2), 569–583. https://doi.org/10.1007/s00216-009-3310-y

Dabrowska, H., Kopko, O., Lehtonen, K. K., Lang, T., Waszak, I., Balode, M., & Strode, E. (2017). An integrated assessment of pollution and biological effects in flounder, mussels and sediment in the southern Baltic Sea coastal area. *Environmental Science and Pollution Research*

*International*, *24*(4), 3626–3639. https://doi.org/10.1007/s11356-016-8117-8

D'Agostini, F., & La Maestra, S. (2021). Micronuclei in Fish Erythrocytes as Genotoxic Biomarkers of Water Pollution: An Overview. In P. de Voogt (Ed.), *Reviews of Environmental Contamination and Toxicology Volume 258* (Vol. 258, pp. 195–240). Springer International Publishing. https://doi.org/10.1007/398_2021_76

Davies, I. M. and Vethaak, A. D. (Ed.). (2012). *Integrated marine environmental monitoring of chemicals and their effects* (No. ICES Cooperative Research Report. Vol 315. 289 pp). https://doi.org/10.17895/ices.pub.5403

Dévier, M.-H., Le Dû-Lacoste, M., Akcha, F., Morin, B., Peluhet, L., Le Menach, K., Burgeot, T., & Budzinski, H. (2013). Biliary PAH metabolites, EROD activity and DNA damage in dab (*Limanda limanda*) from Seine Estuary (France). *Environmental Science and Pollution Research International*, *20*(2), 708–722. https://doi.org/10.1007/s11356-012-1345-7

Díaz-Garduño, B., Perales, J. A., Biel-Maeso, M., Pintado-Herrera, M. G., Lara-Martin, P. A., Garrido-Pérez, C., & Martín-Díaz, M. L. (2018). Biochemical responses of *Solea senegalensis* after continuous flow exposure to urban effluents. *The Science of the Total Environment*, *615*, 486–497. https://doi.org/10.1016/j.scitotenv.2017.09.304

Dolcetti, L., & Venier, P. (2002). Susceptibility to genetic damage and cell types in Mediterranean mussels. *Marine Environmental Research*, *54*(3-5), 487–491. https://doi.org/10.1016/s0141-1136(02)00142-3

Eriksson Wiklund, A.-K., & Dag Broman, B. S. (2005). Toxicity evaluation by using intact sediments and sediment extracts. *Marine Pollution Bulletin*, *50*(6), 660–667. https://doi.org/10.1016/j.marpolbul.2005.02.030

Escartín, E., & Porte, C. (1997). The use of cholinesterase and carboxylesterase activities from *Mytilus galloprovincialis* in pollution monitoring. *Environmental Toxicology and Chemistry*, *16*(10), 2090–2095. https://doi.org/10.1002/etc.5620161015

Evrard, E., Devaux, A., Bony, S., Burgeot, T., Riso, R., Budzinski, H., Le Du, M., Quiniou, L., & Laroche, J. (2010). Responses of the European flounder *Platichthys flesus* to the chemical stress in estuaries: load of contaminants, gene expression, cellular impact and growth rate. *Biomarkers: Biochemical Indicators of Exposure, Response, and Susceptibility to Chemicals*, *15*(2), 111–127. https://doi.org/10.3109/13547500903315598

Fernandes, D., Pujol, S., Pérez-Albaladejo, E., Tauler, R., Bebianno, M. J., & Porte, C. (2014). Characterization of the environmental quality of sediments from two estuarine systems based on different in-vitro bioassays. *Marine Environmental Research*, *96*, 127–135. https://doi.org/10.1016/j.marenvres.2013.09.019

Ford, A. T., Fernandes, T. F., Rider, S. A., Read, P. A., Robinson, C. D., & Davies, I. M. (2003). Reproduction in the amphipod, Echinogammarus marinus: a comparison between normal and intersex specimens. *Journal of the Marine Biological Association of the United Kingdom*, *83*(5), 937–940. https://doi.org/10.1017/S0025315403008099h

Franco, M. E., Johanning, K., Matson, C. W., & Lavado, R. (2022). Reduced biotransformation of polycyclic aromatic hydrocarbons (PAHs) in pollution-adapted Gulf killifish (*Fundulus grandis*). *The Science of the Total Environment*, *806*(Pt 4), 150854. https://doi.org/10.1016/j.scitotenv.2021.150854

Fulton, M. H., & Key, P. B. (2001). Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environmental Toxicology and Chemistry*, *20*(1), 37–45. https://doi.org/10.1002/etc.5620200104

Galloway, T. S., Millward, N., Browne, M. A., & Depledge, M. H. (2002). Rapid assessment of organophosphorous/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. *Aquatic Toxicology (Amsterdam, Netherlands)*, *61*(3-4), 169–180. https://doi.org/10.1016/s0166-445x(02)00051-6

Garcia-Velasco, N., Carrero, J. A., Urionabarrenetxea, E., Doni, L., Zaldibar, B., Izagirre, U., & Soto, M. (2023). Innovative in vivo and in vitro bioassays for the establishment of toxicity thresholds of pollutants in sediment quality assessment using polychaetes and their immune cells. *Chemosphere*, *311*(Pt 1), 136935. https://doi.org/10.1016/j.chemosphere.2022.136935

Garmendia, L., Soto, M., Vicario, U., Kim, Y., Cajaraville, M. P., & Marigómez, I. (2011). Application of a battery of biomarkers in mussel digestive gland to assess long-term effects of

the Prestige oil spill in Galicia and Bay of Biscay: tissue-level biomarkers and histopathology. *Journal of Environmental Monitoring: JEM*, *13*(4), 915–932. https://doi.org/10.1039/c0em00410c

Goksøyr, A. (1995). Use of cytochrome P450 1A (CYP1A) in fish as a biomarker of aquatic pollution. *Archives of Toxicology. Supplement*, *17*, 80–95. https://doi.org/10.1007/978-3-642-79451-3_7

Goksøyr, A., & Förlin, L. (1992). The cytochrome P-450 system in fish, aquatic toxicology and environmental monitoring. *Aquatic Toxicology (Amsterdam, Netherlands)*, *22*(4), 287–311. https://doi.org/10.1016/0166-445x(92)90046-p

Goksøyr, S. Ø., Sørensen, H., Grøsvik, B. E., Pampanin, D. M., Goksøyr, A., & Karlsen, O. A. (2021). Toxicity assessment of urban marine sediments from Western Norway using a battery of stress-activated receptors and cell-based bioassays from fish. *Environmental Toxicology and Pharmacology*, *87*(103704), 103704. https://doi.org/10.1016/j.etap.2021.103704

Gorokhova, E., Martella, G., Motwani, N.H., Tretyakova, N.Y., Sundelin, B., Motwani, H.V., 2020. DNA epigenetic marks are linked to embryo aberrations in amphipods. Scientific Reports 10. https://doi.org/10.1038/s41598-020-57465-1

Grandjean, P., & Landrigan, P. J. (2014). Neurobehavioural effects of developmental toxicity. *Lancet Neurology*, *13*(3), 330–338. https://doi.org/10.1016/S1474-4422(13)70278-3

Hamza-Chaffai, A., Roméo, M., Gnassia-Barelli, M., & El Abed, A. (1998). Effect of copper and lindane on some biomarkers measured in the clam *Ruditapes decussatus*. *Bulletin of Environmental Contamination and Toxicology*, *61*(3), 397–404. https://doi.org/10.1007/s001289900776

Hansson, T., Baršienė, J., Tjärnlund, U., Åkerman, G., Linderoth, M., Zebühr, Y., Sternbeck, J., Järnberg, U., & Balk, L. (2014). Cytological and biochemical biomarkers in adult female perch (*Perca fluviatilis*) in a chronically polluted gradient in the Stockholm recipient (Sweden). *Marine Pollution Bulletin*, *81*(1), 27–40. https://doi.org/10.1016/j.marpolbul.2014.03.001

Hawkins, W. E., Overstreet, R. M., Fournie, J. W., & Walker, W. W. (1985). Development of aquarium fish models for environmental carcinogenesis: tumor induction in seven species. *Journal of Applied Toxicology: JAT*, *5*(4), 261–264. https://doi.org/10.1002/jat.2550050408

HELCOM. (2018). *State Baltic Sea - Second HELCOM holistic assessment 2011-2016. Baltic Sea Environment Proceedings 155* (State of the Baltic Sea – Second HELCOM holistic assessment 2011-2016. Baltic Sea Environment Proceedings (ed.)).

HELCOM. (2023a). *State Baltic Sea. Third HELCOM holistic assessment 2016-2021. Baltic Sea Environment Proceedings n°194*.

HELCOM. (2023b). Reproductive disorders: malformed embryos of amphipods. HELCOM supplementary indicator report; https://indicators.helcom.fi/indicator/reproductive-disorders/.

Hinton, D. E., Kullman, S. W., Hardman, R. C., Volz, D. C., Chen, P.-J., Carney, M., & Bencic, D. C. (2005). Resolving mechanisms of toxicity while pursuing ecotoxicological relevance? *Marine Pollution Bulletin*, *51*(8-12), 635–648. https://doi.org/10.1016/j.marpolbul.2005.07.020

Holth, T. F., & Tollefsen, K. E. (2012). Acetylcholine esterase inhibitors in effluents from oil production platforms in the North Sea. *Aquatic Toxicology (Amsterdam, Netherlands)*, *112-113*, 92–98. https://doi.org/10.1016/j.aquatox.2011.10.019

Hylland, K., Bellas, J., Giltrap, M., & Brooks, S. (2021). *ICES Viewpoint background document: How can we quantify and manage the impact of chemical pollution in the oceans?* https://doi.org/10.17895/ices.pub.5446

Hylland, K., Burgeot, T., Martínez-Gómez, C., Lang, T., Robinson, C. D., Svavarsson, J., Thain, J. E., Vethaak, A. D., & Gubbins, M. J. (2017a). How can we quantify impacts of contaminants in marine ecosystems? The ICON project. *Marine Environmental Research*, *124*, 2–10. https://doi.org/10.1016/j.marenvres.2015.11.006

Hylland, K., Skei, B. B., Brunborg, G., Lang, T., Gubbins, M. J., le Goff, J., & Burgeot, T. (2017b). DNA damage in dab (Limanda limanda) and haddock (*Melanogrammus aeglefinus*) from European seas. *Marine Environmental Research*, *124*, 54–60. https://doi.org/10.1016/j.marenvres.2016.01.001

Hylland, K., Tollefsen, K.-E., Ruus, A., Jonsson, G., Sundt, R. C., Sanni, S., Røe Utvik, T. I., Johnsen, S., Nilssen, I., Pinturier, L., Balk, L., Barsiene, J., Marigòmez, I., Feist, S. W., & Børseth, J. F. (2008). Water column monitoring near oil installations in the North Sea 2001-2004. *Marine*

*Pollution Bulletin*, *56*(3), 414–429. https://doi.org/10.1016/j.marpolbul.2007.11.004

Hylland, K., Vethaak, A. D., Martínez-Gómez, C., Burgeot, T., Svavarsson, J., Gubbins, M. J., McIntosh, A. D., & Thain, J. (2010). Integrated marine contaminant monitoring in the North Sea (ICON): a framework for integrated chemical and biological monitoring. *Marine Environmental Research, 224.*

ICES. (2011). Report of the Study Group on Integrated Monitoring of Contaminants and Biological Effects (SGIMC). *14–18 March 2011, Copenhagen, Denmark. ICES CM 2011/ACOM:30. 265 Pp.* https://doi.org/10.17895/ices.pub.19280786.

Jacobson, T., Sundelin, B., Yang, G., Ford, A.T., 2011. Low dose TBT exposure decreases amphipod immunocompetence and reproductive fitness. Aquat. Toxicol. 101, 72–77. https://doi.org/10.1016/j.aquatox.2010.09.001

Kammann, U., Askem, C., Dabrowska, H., Grung, M., Kirby, M. F., Koivisto, P., Lucas, C., McKenzie, M., Meier, S., Robinson, C., Tairova, Z. M., Tuvikene, A., Vuorinen, P. J., & Strand, J. (2013). Interlaboratory Proficiency Testing for Measurement of the Polycyclic Aromatic Hydrocarbon Metabolite 1-Hydroxypyrene in Fish Bile for Marine Environmental Monitoring. *Journal of AOAC International*, *96*(3), 635–641. https://doi.org/10.5740/jaoacint.12-080

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*(11), 1579–1587. https://doi.org/10.1016/j.chemosphere.2004.11.027

Kolesova, N., Sildever, S., Strode, E., Berezina, N., Sundelin, B., Lips, I., Kuprijanov, I., Buschmann, F., & Gorokhova, E. (2024). Linking contaminant exposure to embryo aberrations in sediment-dwelling amphipods: a multi-basin field study in the Baltic Sea. *Ecological Indicators*, *160*(111837), 111837. https://doi.org/10.1016/j.ecolind.2024.111837

Kopecka, J., Lehtonen, K. K., Barsiene, J., Broeg, K., Vuorinen, P. J., Gercken, J., & 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*(8-9), 406–421. https://doi.org/10.1016/j.marpolbul.2006.03.008

Kopko, O., & Dabrowska, H. (2018). Variability of biological indices, biomarkers, and organochlorine contaminants in flounder (Platichthys flesus) in the Gulf of Gdańsk, southern Baltic Sea. *Chemosphere*, *194*, 701–713. https://doi.org/10.1016/j.chemosphere.2017.12.039

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 (London, England)*, *14*(4), 449–463. https://doi.org/10.1007/s10646-004-1350-9

Kuprijanov, I., Buhhalko, N., Eriksson, U., Sjöberg, V., Rotander, A., Kolesova, N., Lipp, M., Buschmann, F., Hashmi, A., Liblik, T., & Lehtonen, K. K. (2024). A case study on microlitter and chemical contaminants: Assessing biological effects in the southern coast of the Gulf of Finland (Baltic sea) using the mussel *Mytilus trossulus* as a bioindicator. *Marine Environmental Research*, *199*(106628), 106628. https://doi.org/10.1016/j.marenvres.2024.106628

Lacaze, E., Devaux, A., Mons, R., Bony, S., Garric, J., Geffard, A., & Geffard, O. (2011). DNA damage in caged Gammarus fossarum amphipods: a tool for freshwater genotoxicity assessment. *Environmental Pollution (Barking, Essex: 1987)*, *159*(6), 1682–1691. https://doi.org/10.1016/j.envpol.2011.02.038

Lacaze, E., Geffard, O., Goyet, D., Bony, S., Devaux, A., 2011. Linking genotoxic responses in *Gammarus fossarum* germ cells with reproduction impairment, using the Comet assay. Environmental Research 111, 626–634. https://doi.org/10.1016/j.envres.2011.03.012

Lang, T., Wosniok, W., Barsiene, J., Broeg, K., Kopecka, J., & Parkkonen, J. (2006). Liver histopathology in Baltic flounder (*Platichthys flesus*) as indicator of biological effects of contaminants. *Marine Pollution Bulletin*, *53*(8-9), 488–496. https://doi.org/10.1016/j.marpolbul.2005.11.008

Larcher, T., Perrichon, P., Vignet, C., Ledevin, M., Le Menach, K., Lyphout, L., Landi, L., Clerandeau, C., Lebihanic, F., Ménard, D., Burgeot, T., Budzinski, H., Akcha, F., Cachot, J., & Cousin, X. (2014). Chronic dietary exposure of zebrafish to PAH mixtures results in carcinogenic but not genotoxic effects. *Environmental Science and Pollution Research International*, *21*(24),

13833–13849. https://doi.org/10.1007/s11356-014-2923-7
Lastumäki, A., Turja, R., Brenner, M., Vanninen, P., Niemikoski, H., Butrimavičienė, L., Stankevičiūtė, M., & Lehtonen, K. K. (2020). Biological effects of dumped chemical weapons in the Baltic Sea: A multi-biomarker study using caged mussels at the Bornholm main dumping site. *Marine Environmental Research*, *161*, 105036. https://doi.org/10.1016/j.marenvres.2020.105036
Lehtonen, K. K., Ahvo, A., Jørgensen, K. S., Schultz, E., Berezina, N., & Breitholtz, M. (2018). Sediment biotesting in the Baltic Sea: The CONTEST Project. *Nordic Council of Ministers*.
Lehtonen, K. K., d'Errico, G., Korpinen, S., Regoli, F., Ahkola, H., Kinnunen, T., & Lastumäki, A. (2019). Mussel caging and the weight of evidence approach in the assessment of chemical contamination in coastal waters of Finland (Baltic sea). *Frontiers in Marine Science*, *6*, 688. https://doi.org/10.3389/fmars.2019.00688
Lehtonen, K. K., Kankaanpää, H., Leiniö, S., Sipiä, V. O., Pflugmacher, S., & 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 (Amsterdam, Netherlands)*, *64*(4), 461–476. https://doi.org/10.1016/s0166-445x(03)00101-2
Lehtonen, K. K., Schiedek, D., Köhler, A., Lang, T., Vuorinen, P. J., Förlin, L., Barsiene, J., Pempkowiak, J., & Gercken, J. (2006). The BEEP project in the Baltic Sea: overview of results and outline for a regional biological effects monitoring strategy. *Marine Pollution Bulletin*, *53*(8-9), 523–537. https://doi.org/10.1016/j.marpolbul.2006.02.008
Lehtonen, K. K., Turja, R., Budzinski, H., & Devier, M.-H. (2016). An integrated chemical-biological study using caged mussels (*Mytilus trossulus*) along a pollution gradient in the Archipelago Sea (SW Finland, Baltic Sea). *Marine Environmental Research*, *119*, 207–221. https://doi.org/10.1016/j.marenvres.2016.06.003
Lekube, X., Izagirre, U., Soto, M., & Marigómez, I. (2014). Lysosomal and tissue-level biomarkers in mussels cross-transplanted among four estuaries with different pollution levels. *The Science of the Total Environment*, *472*, 36–48. https://doi.org/10.1016/j.scitotenv.2013.10.075
Lowe, D. M., Soverchia, C., & Moore, M. N. (1995). Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. *Aquatic Toxicology (Amsterdam, Netherlands)*, *33*(2), 105–112. https://doi.org/10.1016/0166-445x(95)00015-v
Löf, M., Sundelin, B., Liewenborg, B., Bandh, C., Broeg, K., Schatz, S., Gorokhova, E., 2016a. Biomarker-enhanced assessment of reproductive disorders in *Monoporeia affinis* exposed to contaminated sediment in the Baltic Sea. Ecological Indicators 63, 187–195. https://doi.org/10.1016/j.ecolind.2015.11.024
Marigómez, I., & Baybay-Villacorta, L. (2003). Pollutant-specific and general lysosomal responses in digestive cells of mussels exposed to model organic chemicals. *Aquatic Toxicology (Amsterdam, Netherlands)*, *64*(3), 235–257. https://doi.org/10.1016/s0166-445x(03)00056-0
Marigómez, I., Zorita, I., Izagirre, U., Ortiz-Zarragoitia, M., Navarro, P., Etxebarria, N., Orbea, A., Soto, M., & Cajaraville, M. P. (2013). Combined use of native and caged mussels to assess biological effects of pollution through the integrative biomarker approach. *Aquatic Toxicology (Amsterdam, Netherlands)*, *136-137*, 32–48. https://doi.org/10.1016/j.aquatox.2013.03.008
Martínez-Gómez, C., Bignell, J., & Lowe, D. (2015). Lysosomal membrane stability in mussels. *ICES Techniques in Marine Environmental Sciences No. 56. 41 Pp*. https://doi.org/10.17895/ices.pub.5084
Martínez-Gómez, C., Fernández, B., Robinson, C. D., Campillo, J. A., León, V. M., Benedicto, J., Hylland, K., & Vethaak, A. D. (2017). Assessing environmental quality status by integrating chemical and biological effect data: The Cartagena coastal zone as a case. *Marine Environmental Research*, *124*, 106–117. https://doi.org/10.1016/j.marenvres.2016.04.008
Martínez-Gómez, C., Valdehita, A., Vethaak, A. D., Navas, J. M., & León, V. M. (2020). Toxicity characterization of surface sediments from a Mediterranean coastal lagoon. *Chemosphere*, *253*(126710), 126710. https://doi.org/10.1016/j.chemosphere.2020.126710
Matozzo, V., Tomei, A., & Marin, M. G. (2005). Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. *Marine Pollution Bulletin*, *50*(12), 1686–1693. https://doi.org/10.1016/j.marpolbul.2005.07.011
Monteiro, M., Quintaneiro, C., Nogueira, A. J. A., Morgado, F., Soares, A. M. V. M., & Guilhermino,

L. (2007). Impact of chemical exposure on the fish *Pomatoschistus microps* Krøyer (1838) in estuaries of the Portuguese Northwest coast. *Chemosphere*, *66*(3), 514–522. https://doi.org/10.1016/j.chemosphere.2006.05.061

Moore, M., Lowe, D., & Köhler, A. (2004). Measuring lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences. *ICES Techniques in Marine Environmental Sciences*, *36*, 1–31. https://doi.org/10.17895/ices.pub.5060

Moore, M. N. (1990). Lysosomal cytochemistry in marine environmental monitoring. *The Histochemical Journal*, *22*(4), 187–191. https://doi.org/10.1007/bf02386003

Moore, M. N., Icarus Allen, J., & McVeigh, A. (2006). Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. *Marine Environmental Research*, *61*(3), 278–304. https://doi.org/10.1016/j.marenvres.2005.10.005

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*). *Human and Ecological Risk Assessment: HERA*, *9*(1), 67–94. https://doi.org/10.1080/713609853

Oehlmann, J. (2004). Biological effects of contaminants: Use of intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution. In *ICES Techniques in Marine Environmental Science* (Vol. 37). ICES. https://doi.org/10.17895/ICES.PUB.5061

Oehlmann, J., Fioroni, P., Stroben, E., & Markert, B. (1996). Tributyltin (TBT) effects on *Ocinebrina aciculata* (Gastropoda: Muricidae): imposex development, sterilization, sex change and population decline. *The Science of the Total Environment*, *188*(2-3), 205–223. https://doi.org/10.1016/0048-9697(96)05173-x

Olivares-Rubio, H. F., & Espinosa-Aguirre, J. J. (2021). Acetylcholinesterase activity in fish species exposed to crude oil hydrocarbons: A review and new perspectives. *Chemosphere*, *264*(Pt 1), 128401. https://doi.org/10.1016/j.chemosphere.2020.128401

OSPAR. (1997). *JAMP Guidelines for General Biological Effects Monitoring. Technical Annex 10—reproductive success in fish. OSPAR Commission, London*.

OSPAR. (1998). *JAMP guidelines for general biological effects monitoring. Joint Assessment and Monitoring Programme. OSPAR Commission, London (1998),* 38 p.

OSPAR. (2008). *JAMP Guidelines for Contaminant-specific Biological Effects (OSPAR Agreement 2008-09)*. Oslo and Paris Commissions (2008), 48 p.

OSPAR. (2010). *Quality Status Report 2010*. OSPAR Commission, London, 176 pp.

OSPAR. (2013). Technical Annex on Supporting Parameters for Biological Effects Measurements in Fish and Mussels. Background Document and Technical Annexes for Biological Effects Monitoring, Update 2013. *978-1-909159-22-8 (2013), Pp. 211-219 Monitoring and Assessment Series 589/2013*.

OSPAR. (2023). *Quality Status Report for the North-East Atlantic*. OSPAR Commission, London.

Owen, R., Buxton, L., Sarkis, S., Toaspern, M., Knap, A., & Depledge, M. (2002). An evaluation of hemolymph cholinesterase activities in the tropical scallop, *Euvola (Pecten) ziczac*, for the rapid assessment of pesticide exposure. *Marine Pollution Bulletin*, *44*(10), 1010–1017. https://doi.org/10.1016/s0025-326x(02)00139-x

Pérez-Albaladejo, E., Rizzi, J., Fernandes, D., Lille-Langøy, R., Karlsen, O. A., Goksøyr, A., Oros, A., Spagnoli, F., & Porte, C. (2016). Assessment of the environmental quality of coastal sediments by using a combination of in vitro bioassays. *Marine Pollution Bulletin*, *108*(1-2), 53–61. https://doi.org/10.1016/j.marpolbul.2016.04.063

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., & Regoli, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere*, *83*(4), 475–485. https://doi.org/10.1016/j.chemosphere.2010.12.064

Regoli, F., Frenzilli, G., Bocchetti, R., Annarumma, F., Scarcelli, V., Fattorini, D., & Nigro, M. (2004). Time-course variations of oxyradical metabolism, DNA integrity and lysosomal stability in mussels, *Mytilus galloprovincialis*, during a field translocation experiment. *Aquatic Toxicology (Amsterdam, Netherlands)*, *68*(2), 167–178. https://doi.org/10.1016/j.aquatox.2004.03.011

Repetto, G., Peso, A., & Zurita, J. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, *3*, 1125–1131. https://doi.org/10.1038/nprot.2008.75

Richardson, D. M., Davies, I. M., Moffat, C. F., Pollard, P., & Stag, R. M. (2001). Biliary PAH metabolites and EROD activity in flounder (*Platichthys flesus*) from a contaminated estuarine environment. *Journal of Environmental Monitoring: JEM*, *3*(6), 610–615. https://doi.org/10.1039/b106353g

Richardson, D. M., Gubbins, M. J., Davies, I. M., Moffat, C. F., & Pollard, P. M. (2004). Effects of feeding status on biliary PAH metabolite and biliverdin concentrations in plaice (*Pleuronectes platessa*). *Environmental Toxicology and Pharmacology*, *17*(2), 79–85. https://doi.org/10.1016/j.etap.2004.03.003

Rickwood, C. J., & 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 (Amsterdam, Netherlands)*, *67*(1), 45–56. https://doi.org/10.1016/j.aquatox.2003.11.004

Robinson, C. D., Abbott, L. A., Gubbins, M., Dymond, P., Dobson, J., Thain, J., Davies, I. M., Time Bean, & Bignell, J. (2010). *Integration of passive sampling and mussels monitoring for environmental assessment of Scottish inshore waters*. 2010 Annual Science Conference, Nantes, France. CM 2010/F:19. ASC 2010 - Theme session F. https://doi.org/10.17895/ICES.PUB.25069262

Roche, H., Buet, A., & Ramade, F. (2002). Accumulation of lipophilic microcontaminants and biochemical responses in eels from the Camargue Biosphere Reserve. *Ecotoxicology (London, England)*, *11*(3), 155–164. https://doi.org/10.1023/a:1015418714492

Roubeix, V., Wessel, N., Akcha, F., Aminot, Y., Briaudeau, T., Burgeot, T., Chouvelon, T., Izagirre, U., Munschy, C., & Mauffret, A. (2023). Differences in biomarker responses and chemical contamination among three flatfish species in the Bay of Seine (NE Atlantic). *Marine Pollution Bulletin*, *197*, 115674. https://doi.org/10.1016/j.marpolbul.2023.115674

Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P. J., Dedonyte, 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*(8-9), 387–405. https://doi.org/10.1016/j.marpolbul.2005.11.013

Schnell, S., Olivares, A., Piña, B., Echavarri-Erasun, B., Lacorte, S., & Porte, C. (2013). The combined use of the PLHC-1 cell line and the recombinant yeast assay to assess the environmental quality of estuarine and coastal sediments. *Marine Pollution Bulletin*, *77*(1-2), 282–289. https://doi.org/10.1016/j.marpolbul.2013.09.047

Soreq, H., & Seidman, S. (2001). Acetylcholinesterase — new roles for an old actor. *Nature Reviews. Neuroscience*, *2*, 294–302. https://doi.org/10.1038/35067589

Stankevičiūtė, M., Gomes, T., & González, J. A. C. (2022). *Nuclear abnormalities in mussel haemocytes and fish erythrocytes*. ICES Techniques in Marine Environmental Science. Vol 66 13 pp. https://doi.org/10.17895/ices.pub.21220031

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. *Marine Environmental Research*, *55*(2), 137–159. https://doi.org/10.1016/s0141-1136(02)00212-x

Strode, E., Jansons, M., Purina, I., Balode, M., & Berezina, N. A. (2017). Sediment quality assessment using survival and embryo malformation tests in amphipod crustaceans: The Gulf of Riga, Baltic Sea AS case study. *Journal of Marine Systems: Journal of the European Association of Marine Sciences and Techniques*, *172*, 93–103. https://doi.org/10.1016/j.jmarsys.2017.03.010

Sturve, J., Hultman, M. T., Wassmur, B., & Almroth, B. C. (2017). Determining oxidative stress and EROD activity in dab (*Limanda limanda*) in the North and Baltic Seas. *Marine Environmental Research*, *124*, 46–53. https://doi.org/10.1016/j.marenvres.2016.10.008

Sundelin, B., & Eriksson, A. K. (1998). Malformations in embryos of the deposit-feeding amphipod Monoporeia affinis in the Baltic Sea. *Marine Ecology Progress Series*, *171*, 165–180. https://doi.org/10.3354/meps171165

Sundelin, B., Eriksson Wiklund, A.-K., & Ford, A. T. (2008). *Biological effects of contaminants: the*

*use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors*. ICES Techniques in Marine Environmental Science, Vol. 41. https://doi.org/10.17895/ices.pub.5065

Sussarellu, R., Chouvelon, T., Aminot, Y., Couteau, J., Loppion, G., Dégremont, L., Lamy, J.-B., Akcha, F., Rouxel, J., Berthelin, C., Briaudeau, T., Izagirre, U., Mauffret, A., Grouhel, A., & Burgeot, T. (2022). Differences in chemical contaminants bioaccumulation and ecotoxicology biomarkers in *Mytilus edulis* and *Mytilus galloprovincialis* and their hybrids. *Environmental Pollution (Barking, Essex: 1987)*, *292*(Pt A), 118328. https://doi.org/10.1016/j.envpol.2021.118328

Tairova, Z. M., Christensen, J. P. A., & Strand, J. (2024). Reproductive disorders in amphipods as indicators of effects of hazardous substances. *Reproduction*. https://dce.au.dk/fileadmin/dce.au.dk/Udgivelser/Notater_2024/N2024_10.pdf

Tairova, Z., & Strand, J. (2022). Biological effect measurements in *Gammarus spp*. and *Corophium volutator* as indicators of toxic effects of hazardous substances in Danish coastal waters. *Aarhus University, DCE – Danish Centre for Environment and Energy, 24 Pp. Technical Report No. 237*. http://dce2.au.dk/pub/TR237.pdf

Thomas, K. V., Balaam, J., Chan-Man, Y., Hurst, M. R., & Thain, J. E. (2004). *The Use Of In Vitro Bioassays And Bioassay-Directed Analysis/Tie In Marine Ecosystem Management*. ICES Annual Science Conference, Vigo, Spain. CM 2004/Z:01. https://doi.org/10.17895/ices.pub.25349962

Turja, R., Benito, D., Ahvo, A., Izagirre, U., Lekube, X., Stankevičiūtė, M., Butrimavičienė, L., Soto, M., & Lehtonen, K. K. (2023). Biomarker responses in mussels (Mytilus trossulus) from the Baltic Sea exposed to water-accommodated fraction of crude oil and a dispersant at different salinities. *Marine Pollution Bulletin*, *192*, 115100. https://doi.org/10.1016/j.marpolbul.2023.115100

Turja, R., Sanni, S., Stankevičiūtė, M., Butrimavičienė, L., Devier, M.-H., Budzinski, H., & Lehtonen, K. K. (2020). Biomarker responses and accumulation of polycyclic aromatic hydrocarbons in *Mytilus trossulus* and *Gammarus oceanicus* during exposure to crude oil. *Environmental Science and Pollution Research International*, *27*(13), 15498–15514. https://doi.org/10.1007/s11356-020-07946-7

Valbonesi, P., Sartor, G., & Fabbri, E. (2003). Characterization of cholinesterase activity in three bivalves inhabiting the North Adriatic sea and their possible use as sentinel organisms for biosurveillance programmes. *The Science of the Total Environment*, *312*(1-3), 79–88. https://doi.org/10.1016/S0048-9697(03)00227-4

Vethaak, A. D., Davies, I. M., Thain, J. E., Gubbins, M. J., Martínez-Gómez, C., Robinson, C. D., Moffat, C. F., Burgeot, T., Maes, T., Wosniok, W., Giltrap, M., Lang, T., & Hylland, K. (2017). Integrated indicator framework and methodology for monitoring and assessment of hazardous substances and their effects in the marine environment. *Marine Environmental Research*, *124*, 11–20. https://doi.org/10.1016/j.marenvres.2015.09.010

Viarengo, A., Lowe, D., Bolognesi, C., Fabbri, E., & Koehler, A. (2007). The use of biomarkers in biomonitoring: a 2-tier approach assessing the level of pollutant-induced stress syndrome in sentinel organisms. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *146*(3), 281–300. https://doi.org/10.1016/j.cbpc.2007.04.011

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*(10), 1416–1427. https://doi.org/10.1016/j.chemosphere.2009.06.005

Wessel, N., Santos, R., Menard, D., Le Menach, K., Buchet, V., Lebayon, N., Loizeau, V., Burgeot, T., Budzinski, H., Akcha, F. (2010). Relationship between PAH biotransformation as measured by biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (Solea solea). Marine Environmental Research 69, S71–S73. https://doi.org/10.1016/j.marenvres.2010.03.004

WGBEC. (2010). *Report of the Working Group on Biological Effects of Contaminants (WGBEC), Dublin, 11- 15 January* (No. ICES CM 2010/SSGHIE:01).

Zinkl, J. G., Lockhart, W. L., Kenny, S. A., & Ward, F. J. (1991). *The effects of cholinesterase inhibiting insecticides on fish*. https://doi.org/10.5555/19930514906

# Annex A – biomarker description

A range of biomarkers have been found to respond to contaminant exposure in specific studies or in field studies but have not been recommended in this guideline (Table S1). Furthermore, some methods are seen as promising but need further development. Further description of the biomarkers and motivation can be found below (Table S2).

**Table S1.** Biomarkers that have been used in previous or ongoing monitoring programmes but have not been selected in this guideline.

| Mechanism of toxicity | Biomarker | J | H | S | W | |
|---|---|---|---|---|---|---|
| **developmental toxicity** | embryonal development eelpout | X | X | | X | |
| **endocrine disruption** | intersex | | | X | | |
| **genotoxicity** | DNA adducts in liver | X | | | X | fish |
| **membrane disruption** | lysosomal membrane stability (LMS) – cytochemical | X | X | X | X | |
| **metal toxicity** | metallothionein concentration in liver | X | | | | |
| **metal toxicity** | ALA-D inhibition in red blood cells | X | | | X | |
| **general health** | externally visible fish diseases | | | X | | |
| **growth, energetics** | whole organism (SfG) / clearance rate | | | X* | X | |
| | Stress on Stress | | | X | | |
| **membrane disruption** | lysosomal membrane stability (LMS) - NRR | | X | X | X | bivalves |
| **metal toxicity** | metallothionein concentration in digestive gland | | | | X | |

J: JAMP/preCEMP; H: HELCOM (C; S: SGIMC (OSPAR 2013), W: ICES WGBEC

* only part of the analysis was recommended (clearance rate)

In addition to the overview of specific methods in tables S1 and S2, metabolomics, proteomics, transcriptomics epigenomics comprise a range of analytical techniques that are relevant to processes involving contaminants. Their main current use is to identify potential markers. There are no clear strategies to standardise them for use in environmental monitoring programmes.

All omics strategies can both be used to identify and quantify processes in organisms and in a “meta” mode to describe communities, often combined with genomic studies (eDNA). In addition to the above, there is very fragmented knowledge on how contaminants may affect the microbiome of fish or invertebrates. This is clearly an area that merits further research.

# Background and motivation - biomarkers not included in recommendations

### Developmental toxicity - embryonal development (eelpout)

Eelpout (*Zoarces viviparus*) is found from the White Sea to the southern North Sea and has been used for biomonitoring (Neuman et al., 1999; Strand et al., 2004; Strand & Dahllöf, 2005; Tairova et al., 2012). Eelpout has limited migration and is viviparous, making them relevant as a sentinel species for developmental toxicity, as the responses in individual female fish can be linked to larval development and reproductive success (Hedman et al., 2011; Asker at al 2015).

Reproduction in eelpout is a method recommended by ICES, OSPAR and HELCOM and has been found to be affected by endocrine disrupting compounds, among other pollutants (Hedman et al., 2011; Jacobsson et al., 1986; Morthorst et al., 2014).

The assay is performed using samples from sexually mature female eelpout to assess abnormal development in their larvae and embryos, including malformations (skeletal/morphological), larval size (to indicate abnormal growth rates) and number of late dead larvae (Strand Ch 13 in Davies & Vethaak et al., 2012). Sex ratios can also be assessed and has for example shown masculinization in eelpouts exposed to effluents from pulp mills (Larsson & Förlin, 2002).

Although the species has been used for monitoring for many years there is still limited understanding of how important contaminant exposure is compared to e.g. oxygen deficiency, temperature or food availability and there is a scarcity of studies showing dose-response following exposure to contaminants. The species is not found south of the North Sea and has low density in many coastal areas within its range of distribution.

### Endocrine disruption - intersex (fish)

Male fish with the intersex condition have oocytes within the testis; this being regarded as a phenotypic endpoint of endocrine disruption (both natural and anthropogenic) in male fish. Owing to the fact that the testis may appear normal from external observations, histological examination of the testis is necessary to identify and grade individual cases of intersex and to estimate prevalence in a population. The condition does not appear to be relevant to the species currently included in monitoring programmes and has not been recommended.

### Genotoxicity - DNA adducts in liver (fish)

The concentration of DNA adducts gives an indication of genotoxicity. DNA adducts represent a change in the structure of DNA which can lead to misreads and breaks. During metabolism of polycyclic aromatic compounds, reactive intermediates can be formed which are able to bind to nucleic acids and other structures, causing genotoxicity (Dolcetti et al., 2002; Varanasi & Stein, 1991). In addition to PAHs, PCBs are also known to have these genotoxic effects, and cause DNA adducts in fish (e.g. Hylland et al., 2017; Sundberg et al.,

2007). DNA adducts are relatively persistent and provides an indication of both current and past exposure.

DNA adducts can be quantified using the $^{32}$P postlabelling method in a multistep process where DNA samples are first isolated from tissue, then hydrolysed, enriched and enzymatically labelled with radioactive $^{32}$P-ATP to allow mononucleotides modified by xenobiotics to be quantified (Reichert et al., 1999). The assay is non-specific in that different compounds that bind to DNA can be detected (Reichert et al., 1999) and it is also not species specific, allowing a range of fish and bivalves to be used for testing (Ch 8 in Davies and Vethaak 2012).

Analyses of DNA adducts involves using radioactive phosphorus isotopes and is a time-consuming process as well as requiring very specific competence. For these reasons it is expensive. Very few European labs currently perform this analysis.

A promising development is DNA adductomics, particularly through high-resolution mass spectrometry (HRMS). Unlike traditional methods, HRMS-based adductomics allows for the simultaneous detection of multiple adduct classes within a single sample (Balbo et al., 2014). The method has been used for analysis of environmental samples (Gorokhova et al. 2020; Martella et al., 2023).

### Metal toxicity - hepatic metallothionein concentration (fish)

Metallothioneins (MTs) are small, cysteine-rich proteins (Wood et al., 2012) found both in vertebrates and invertebrates. Metallothionein has been used as a biomarker of metal toxicity in both fish and bivalves (see discussion of MT in bivalves below). The physiological role of the protein is mainly to regulate cellular availability of the essential elements copper and zinc, but it also has a role in radical scavenging. Cadmium and mercury are non-essential metals that will bind to and induce metallothionein production in fish (Hylland et al., 2009; Siscar et al., 2014; Fernandes et al., 2008).

Using metallothionein as a marker for toxic metal stress is not straightforward since it is primarily a marker for Cu and Zn metabolism (Hylland, Ch 14 in Davies and Vethaak 2012).

### Metal toxicity - ALA-D inhibition in RBCs (fish)

Delta-aminolevulinic acid dehydratase (ALA-D) is an enzyme involved in the second step of the synthesis of the iron-containing compound haem, which is part of haemoglobin and other proteins. ALA-D is a metal specific effects marker, predominantly sensitive to lead, but its activity may also be affected by exposure to other metals. The enzyme has been considered a good biomarker for lead exposure in fish (Hylland, 2004).

This biomarker has only been used in a few national programmes. A change in protocol (to remove mercury) still needs to be properly assessed. When a satisfactory protocol is available, the biomarker should be more widely used.

### Membrane disruption– lysosomal membrane stability (LMS) in fish liver

Lysosomes play a key role in liver injury in fish caused by organic and inorganic xenobiotics. Based on the cytochemical demonstration of hydrolase activity on frozen liver sections, LMS in liver cells has been applied for assessing the biological effects of chemical pollutants in a variety of fish species (Broeg et al. 1999; Kohler et al. 2002; Alvarado et el., 2005; Einsporn et al. 2005; Schiedek et al. 2005; Sturve et al. 2005; Bilbao et al. 2006; Kopecka et al., 2006; Zorita et al.2007; Izagirre, 2008; Briaudeau et al., 2020, 2021); these include flatfish (e.g., flounder, dab, turbot, sole), eelpout, seabass, red mullet, thick-lip grey mullet and pelagic fishes (e.g. saithe, herring, anchovy, hake).

The histological architecture of the fish liver is complex and varies largely within and between fish species (Akiyosi and Inoue, 2004). High levels of lipid content in fish liver and its natural variability may jeopardise sample processing and LMS quantification (Izagirre, 2008). Results from field studies (Broeg et al. 1999; Einsporn et al. 2005; Schiedek et al. 2005; Sturve et al. 2005; Bilbao et al. 2006; Kopecka et al., 2006; Zorita et al. 2007) do suggest this biomarker as a promising candidate.

### General Health– externally visible disease (fish)

External visible disease was previously included as a biological effect for contaminants indicator. Fish are visually inspected for the presence and severity of nine different diseases or parasites including: lymphocystis; epidermal hyperplasia / papilloma; skin ulcerations; fin rot; x-cell gill disease; hyperpigmentation; *Stephanostomum baccatum; Acanthochondria cornuta; Lepeophtheirus pectoralis* following ICES TIMES 19 (Bucke et al. 1996).

Tthe aetiology of the diseases is multifactorial and other environmental factors are influential including osmotic stress, anaerobic sediments, overcrowding, nutritional deficiencies, seasonal changes, oxygen deficiencies and pollution levels (Mellergaard & Nielsen, 1995; Vethaak, 1992; Vethaak & Jol, 1996; Winzer, Winston, Becker, Van Noorden & Köhler, 2001). This indicator has not been included in the revised guideline for biological effects of contaminant monitoring.

### General health - digestive gland atrophy (bivalves)

The digestive gland in bivalves plays a role in digestion as well as in accumulation and detoxification of pollutants. It is a key target tissue for xenobiotic pollution and is commonly used to assess effects on mussel health after exposure of e.g. biocides, personal care products, microplastics and nanoparticles (Faggio et al., 2018; Courant et al., 2017). The composition of the gland's epithelium can change in response to pollutants which may, in turn, affect the bivalve’s digestion and ability to metabolise pollutants (Marigómez et al., 1998; Dimitriadis et al., 2004; Bignell et al. Ch 15 in Davies & Vethaak 2012 and references therein).

Changes in the relative cell composition of the digestive gland’s epithelial tubules can be assessed by sectioning and staining embedded digestive glands followed by comparing the counts of normal digestive cells to basophilic cells using a stereological procedure (Soto et al., 2002; Bignell et al., Ch 15 in Davies & Vethaak 2012). Histopathological alterations, such

as lesions, can also be assessed in bivalve digestive glands (Costa et al., 2013; Joshy et al., 2020).

There is evidence that contaminant exposure will affect the morphology of digestive gland in mussels, both from laboratory and field studies. Once the current conundrum of different Mytilus species, it would appear this is a biomarker that could be included environmental monitoring, following general acceptance of methods and assessment criteria.

### Growth, energetics - SfG / clearance rate (bivalves)

The physiological biomarker scope for growth (SfG), i.e. the estimated energy available for growth in filter-feeders, has a clear link to potential population impacts. SfG can be used as a marker for sublethal stress caused by xenobiotic exposure (Widdows in Davies & Vethaak 2012; Albentosa et al., 2012). This biomarker has mainly been used for suspension-feeding mussels such as *Mytilus* sp (Widdows & Staff, 2006). The main component in the calculation is "clearance rate", which has been suggested as a less comprehensive biomarker.

### Immunotoxicity (bivalves)

The immune system of bivalves is complex. Robust markers for immunotoxicity for biomonitoring are not available. Compounds with potential immunotoxic effects include heavy metals, PAHs, chlorinated pesticides, pharmaceuticals, nanoparticles and PCBs in invertebrates (Renault, 2015; Shi et al, 2017; Barmo et al., 2013; Ciacci et al., 2013). Inflammation in bivalves can occur to varying degrees following exposure to some xenobiotics and a scoring index is available to assess different severities of inflammation in bivalves (Bignell et al., Ch 15 in Davies & Vethaak 2012). Granulocytoma, or “large foci of inflammation” can be seen in cases of heightened immune response, together with increased haemocyte infiltration and brown cell foci (Bignell et al., Ch 15 in Davies & Vethaak 2012; Costa et al., 2013). Flow cytometry can be used to assess hemocyte counts in mussel hemolymph samples, as hemocytes play an important role in the invertebrate immune system (Parrino et al., 2019; Renwrantz et al., 2013).

### Membrane disruption – lysosomal membrane stability (LMS) - NRR (bivalves)

The neutral red retention (NRR) assay method was initially described for mussels *M. edulis* and *M. galloprovincialis*, by Moore et al. (2004) and updated by Martínez-Gómez et al. (2015). Further, it has also been adapted for Baltic Sea mussels, *M. trossulus* (Turja et al., 2013; Lastumäki et al., 2020).

The NRR assay has been applied in national monitoring programmes for the Mediterranean Sea (Barcelona Convention) and in regional field studies in the North Atlantic, the Baltic Sea and the Mediterranean Sea (Lowe et al., 1995a; Cajaraville et al., 1996; Dallianis et al., 2003, Castro et al., 2004; Martinez-Gomez et al., 2008; Brooks et al., 2011; Turja et al., 2013; Martínez-Gómez et al., 2017; Lastumäki et al., 2020).

One particular challenge remains for this biomarker: lysosomal morphology in hemocytes vary substantially under different conditions. Training technical personnel in the identification of both 'normal' and 'abnormal' granulocyte appearances, and on lysosomal

abnormalities, is essential. Another challenge is the need for the NRR assay to be performed on freshly extracted haemocytes, thus requiring transport of live animals to the laboratory.

### Metal toxicity - metallothionein concentration in hepatopancreas (bivalves)

Metallothionein (MT) in bivalves are involved in the sequestration/binding of metals, possibly with some variation across bivalve species and tissues. Metallothionein both regulates natural processes involving Cu and Zn but may also be induced following exposure to e.g. Cd and Hg (Hylland, Chapter 14 in Davies & Vethaak, 2012). MT in invertebrates exists in different forms, where MT-10 and MT-20 seem to be the main forms in mussels (Aceto et al., 2011).

MTs can be measured in specific bivalve tissues, mainly the hepatopancreas or gill, or in whole mussel soft tissue and are widely used as a biomarker (Le et al., 2016). Different quantification protocols can be used, including the sulfhydryl method (Viarengo et al., 1997), electrochemical differential pulse polarography method (Olafson and Thompson, 1974) or metal substitution.

It can be noted that there can be some variation in MT concentrations within the same samples depending on the method used (Hylland, Chapter 14 in Davies & Vethaak 2012). Furthermore, MT can vary by season and there might also be high natural variability. As a result of this and other inconsistent findings, there are uncertainties tied to the use of MT as a biomarker for metal effects (Le et al., 2016; Davies & Vethaak 2012).

## Promising biomarkers

**Table S2.** Biomarkers needing further development.

| Mechanism of toxicity | Effect method (biomarker) | |
|---|---|---|
| **modulation of biotransformation** | phase II enzymes in liver | fish |
| **cardiotoxicity** | troponin in blood or histological | |
| **endocrine disruption** | thyroid hormones in blood | |
| **immunotoxicity** | cytokines, APR in plasma | |
| **oxidative stress** | protein carbonylation in tissues | |
| **oxidative stress** | lipid peroxidation in tissues | |
| **modulation of biotransformation** | phase II enzymes | bivalves |
| **general health** | digestive gland atrophy | |
| **immunotoxicity** | cytokines, lysozyme, acute phase reactants in plasma | |
| **oxidative stress** | protein carbonylation in tissues | |
| **oxidative stress** | lipid peroxidation in tissues | invertebrates |
| **oxidative stress** | oxidative stress enzymes, antioxidant concentrations | fish, bivalves |

### Modulation of biotransformation - phase II enzymes (fish)

Following biotransformation in phase I, polar molecules are added to the xenobiotic compound in phase II, making the xenobiotic molecules larger, more hydrophilic and thus easier to excrete (Srikanth et al., 2013; George, 1994; Kroon et al., 2017). The main phase II enzyme families in fish and other vertebrates are glutathione S-transferases (GSTs), sulphotransferases (SULTs) and UDP-glucuronyl transferases (UDP-GTs).

Glutathione S-transferases (GSTs) have been widely used as biomarkers in fish tissues, for example after PAH exposure (e.g. Turja et al., 2023; Kopecka et al., 2006). GST catalyses the step where glutathione (GSH) conjugates electrophilic xenobiotic compounds (Townsend & Tew 2003). GSTs also play a crucial role in oxidative stress defences, responding to exposure to metals and other stressors. The ratio between reduced and oxidised glutathione (GSH and GSSG) - glutathione redox status- is used as a marker for fish health and indication of oxidative stress as GSH acts as a redox buffer. Total glutathione has also been used as a marker to examine fish health after xenobiotic exposure (e.g. Benedetti et al., 2012; Piva et al., 2011). GSH is of key importance for the conjugation step of phase II transformation (Kroon et al., 2017), but also the most important antioxidant in cells.

Another important enzyme family involved in phase II detoxification is UDP-glucuronyl transferases (UDPGTs). These enzymes are catalysts for the transfer glucuronic acid to the xenobiotic substances for detoxification and excretion (e.g. Clarke et al., 1992; Schlenk et al., 2008).

In most organisms and tissues, phase-II enzymes are not strongly inducible and their constitutive activity high. Their activity relates to other conditions in cells than contaminant stress since their co-factors are involved in general cell metabolism. Hence they are not generally contaminant-specific. Glutathione *S*-transferases are also involved in oxidative stress. In addition, GST activity is generally determined using a substrate for general activity and there is limited understanding of the role of different GSTs in biotransformation.

### Cardiotoxicity - troponins in blood or heart morphology (fish)

Cardiotoxicity is important toxicity mechanism that has been proposed for biomonitoring, particularly due to the potential for population level impact (Hylland et al., 2021). Heart morphology, the expression of heart-related genes or heart development in fish can for example be affected and disrupted by compounds related to oil production and oil spills, including PAHs which are ubiquitous in the aquatic environment but also by other contaminants (Marris et al., 2020; Huang et al., 2018; Sørhus et al., 2023; Incardona et al., 2015).

Cardiotoxicity can be assessed through functional and anatomical investigation of fish hearts, sometimes combined with alterations in expression of heart-related genes (e.g. Incardona et al., 2021). The mechanism behind cardiotoxicity may for instance be linked to altered action potential and calcium signalling in the fish's heart cells (cardiomyocytes) (Brette et al., 2014).

While the mechanism of toxicity is clearly relevant, there is a need to develop appropriate biomarkers, troponin measurement or standardised morphological assessment, and to clarify the importance of confounding factors, e.g. other sources of stress.

### Endocrine disruption - thyroid hormones in plasma (fish)

While most ecotoxicological studies on endocrine disruption have been focused on reproductive systems and sex steroids, other endocrine systems are also important for the organism's health. After oestrogens and androgens, the most studied endocrine system is the thyroid system, which is vital for growth, and development in vertebrates (Knapen et al., 2020; Mullur et al., 2014; Williams, 2008). In fish, thyroid hormones (THs) are also involved in processes like osmoregulation, larval metamorphosis, energy metabolism and neurodevelopment (Knapen et al., 2020; Deal & Volkoff 2020; Besson et al., 2020; Power et al., 2001; Blanton and Specker, 2007).

Levels of thyroxine (T4) and triiodothyronine (T3) - in fish blood samples have been studied in a range of fish species in both field and laboratory settings. These studies have shown effects of compounds with thyroid disruptive potential like PCBs, PBDEs and PFASs (e.g. Brar et al., 2010; Birgersson et al., 2021). There are still some limitations to the understanding of exactly how pollutants disrupt the TH regulatory system.

Measurements of T3 and T4 levels can be performed using a radioimmunoassay (RIA) with the appropriate antibodies or an ELISA assay. The success of these methods can vary between fish species. For smaller fish like zebrafish larvae, whole animal samples can be used instead of plasma.

### Immunotoxicity (fish)

Several groups of contaminants, including metals, pesticides and endocrine disruptors including PFASs, have the potential to disrupt or suppress the immune system in fish (e.g. Han et al., 2012; Fang et al., 2013; Gao, 2020; Sueiro et al., 2020). Compounds acting as ligands of the aryl hydrocarbon receptor may also act as immunosuppressants in fish (Segner et al., 2021). Many different assays are used for assessments of fish immunotoxicity, including cytokines, lysozyme, acute phase reactants in plasma as well as levels of the immune-related white blood cells, immune-related mRNA expression (Zhu et al., 2013). Considering the complexity of the immune system and the variations between different species (Pedersen and Babayan, 2011), setting joint markers and protocols for assessing immunotoxicity is not straightforward (Rehberger et al., 2017).

Immunotoxicity measurements are still not included in many biomonitoring programmes. Most studies have focused on disruption of innate immune responses and immunosuppression after chemical exposure while other immune disruptions caused by toxicants, such as autoimmunity and hypersensitivity are rarely explored and still need more research (Martínez-Gómez & Vethaak, 2019; Rehberger et al., 2017; Segner et al., 2012).

While clearly an important mechanism of toxicity there are no satisfactory methods available at this time. A research break-through is sorely needed.

### Oxidative stress - protein carbonylation in tissues (fish)

Protein carbonylation refers to the oxidation of proteins, and is used as a biomarker of oxidative stress/oxidative damage to proteins in fish (Dalle-Donne et al., 2003; Shacter et al., 1994). This is an irreversible process and certain amino acid residues are more sensitive to oxidation at their side chains than others (Almroth et al., 2008; Stadtman and Levine, 2000) Increased amounts of oxidised protein is also linked to ageing in fish (e.g. Kishi, 2004).

To assess protein oxidation, carbonyl-specific reagents such as 2, 4-dinitrophenylhydrazine can be used. Protein carbonyl formation can be quantified colorimetrically using an ELISA (Winterbourn & Buss, 1999; Almroth et al., 2008) or by using commercial reagent kits for fluorometric assays (e.g. Vieira et al., 2022).

As for other biomarkers for oxidative stress, the direct link to contaminant exposure is not clear since they are also strongly affected by other factors and depend on other components in the cellular defence against oxidative stress.

### Oxidative stress - lipid peroxidation in tissues (fish)

Lipid peroxidation predominantly occur as a result of oxidative stress, for example from metal exposure, genotoxic compounds and ROS, leading to oxidation of polyunsaturated fatty acids. The concentration of lipid peroxidation can be measured in fish tissues to assess oxidative damage/oxygen toxicity (Kroon et al., 2017). These levels are quantified by measuring degradation products in different tissues (liver, gill, kidney, and brain) and species of fish. Seasonal variations on lipid peroxidation have also been shown (e.g Oliva et al., 2012; Pereira et al., 2010).

Thiobarbituric acid reactive substances (TBARS) protocol can be used to assess peroxidations in fish blood cells, such as determination of the level of malondialdehyde (MDA) in plasma (Costa et al., 2011; Omar et al., 2016).

As for other biomarkers for oxidative stress, the direct link to contaminant exposure is not clear since they are also strongly affected by other factors and depend on other components in the cellular defence against oxidative stress.

### Modulation of biotransformation - phase II enzymes (bivalves)

Our general understanding of biotransformation is less advanced for bivalves than for e.g. fish. While other phase-II enzyme families are present in bivalves, it is predominantly GST that has been used. There are however many uncertainties in terms of regulation related to contaminant-exposure, endogenous confounding factors and the influence of external confounders.

### Oxidative stress - protein carbonylation (bivalves)

Protein carbonylation, the oxidation of proteins, is used as a marker of oxidative stress in both bivalves and fish (see above). During carbonylation, amino acid side chains are irreversibly altered which can cause the proteins to aggregate or be degraded (Levine et al., 2000). Carbonyl-specific reagents like 2.4-dinitrophenylhydrazine (DNPH) (Reznick and Packer 1994) or immunoblotting techniques can be used to assess carbonylation (e.g. Martyniuk et al., 2022; Chora et al., 2010). The protein carbonylation can be tissue-specific, for example, a higher response observed in the gills compared to the digestive gland in response to nanoparticles (Chora et al., 2010).

As referred to above for fish, there are challenges involved in using biomarkers for oxidative stress, the two most important being the complex processes involved in cellular defence and confounding factors.

### Oxidative stress - lipid peroxidation in tissues (invertebrates)

Lipid peroxidation can be used as an indicator of the damage caused by oxidative stress in invertebrates as well as in fish (see above). Quantification of thiobarbiturate reactive substances (TBARS) can be used to measure lipid peroxidation products in mussel gill tissue samples, and is based on malondialdehyde detection, as malondialdehyde is a byproduct of the peroxidation (Gillis et al., 2014; Kwok et al., 2012; Draper & Hadley, 1990). Lipid peroxidation is a transient biomarker, showing ongoing stress from exposure to contaminants such as metals (Dorts et al., 2009; Gillis et al., 2014).

As referred to above, there are challenges involved in using biomarkers for oxidative stress, the two most important being the complex processes involved in cellular defence and confounding factors.

## References


Aceto, S., Formisano, G., Carella, F., De Vico, G., & Gaudio, L. (2011). The metallothionein genes of *Mytilus galloprovincialis*: genomic organization, tissue expression and evolution. *Marine Genomics*, *4*(1), 61–68. https://doi.org/10.1016/j.margen.2011.01.001

Akiyoshi, H., & Inoue, A. (2004). Comparative histological study of teleost livers in relation to phylogeny. *Zoological Science*, *21*(8), 841–850. https://doi.org/10.2108/zsj.21.841

Albentosa, M., Viñas, L., Besada, V., Franco, A., & González-Quijano, A. (2012). First measurements of the scope for growth (SFG) in mussels from a large scale survey in the North-Atlantic Spanish coast. *The Science of the Total Environment*, *435-436*, 430–445. https://doi.org/10.1016/j.scitotenv.2012.07.025

Almroth, B. C., Sturve, J., Stephensen, E., Holth, T. F., & Förlin, L. (2008). Protein carbonyls and antioxidant defenses in corkwing wrasse (*Symphodus melops*) from a heavy metal polluted and a PAH polluted site. *Marine Environmental Research*, *66*(2), 271–277. https://doi.org/10.1016/j.marenvres.2008.04.002

Alvarado, N. E., Buxens, A., Mazón, L. I., Marigómez, I., & Soto, M. (2005). Cellular biomarkers of exposure and biological effect in hepatocytes of turbot (*Scophthalmus maximus*) exposed to Cd, Cu and Zn and after depuration. *Aquatic Toxicology (Amsterdam, Netherlands)*, *74*(2), 110–125. https://doi.org/10.1016/j.aquatox.2005.03.024

Asker, N., Carney Almroth, B., Albertsson, E., Coltellaro, M., Bignell, J. P., Hanson, N., Scarcelli, V., Fagerholm, B., Parkkonen, J., Wijkmark, E., Frenzilli, G., Förlin, L., & Sturve, J. (2015). A gene to organism approach—assessing the impact of environmental pollution in eelpout (*Zoarces viviparus*) females and larvae. *Environmental Toxicology and Chemistry / SETAC*, *34*(7), 1511–1523. https://doi.org/10.1002/etc.2921

Babich, H., & Borenfreund, E. (1992). Neutral red assay for toxicology in vitro. *In Vitro Methods of Toxicology (R. R. Watson, Ed.)*, *CRC Press Boca Raton, FL 17*, 237–251.

Barmo, C., Ciacci, C., Canonico, B., Fabbri, R., Cortese, K., Balbi, T., Marcomini, A., Pojana, G., Gallo, G., & Canesi, L. (2013). In vivo effects of n-TiO2 on digestive gland and immune function of the marine bivalve *Mytilus galloprovincialis*. *Aquatic Toxicology (Amsterdam, Netherlands)*, *132-133*, 9–18. https://doi.org/10.1016/j.aquatox.2013.01.014

Benedetti, M., Ciaprini, F., Piva, F., Onorati, F., Fattorini, D., Notti, A., Ausili, A., & Regoli, F. (2012). A multidisciplinary weight of evidence approach for classifying polluted sediments: Integrating sediment chemistry, bioavailability, biomarkers responses and bioassays. *Environment International*, *38*(1), 17–28. https://doi.org/10.1016/j.envint.2011.08.003

Besson, M., Feeney, W. E., Moniz, I., François, L., Brooker, R. M., Holzer, G., Metian, M., Roux, N., Laudet, V., & Lecchini, D. (2020). Anthropogenic stressors impact fish sensory development and survival via thyroid disruption. *Nature Communications*, *11*(1), 3614. https://doi.org/10.1038/s41467-020-17450-8

Bilbao, E., Soto, M., Cajaraville, M.P., Cancio, I., Marigómez, I. 2006. Cell and tissue-level biomarkers of pollution in wild pelagic fish, herring (*Clupea harengus*) and saithe (*Pollachius virens*), from the North Sea. In: Biological effects of contaminants in marine pelagic ecosystems. (eds: Hylland, K., Lang, T., Vethaak, D.), SETAC Press, Pensacola, FL, USA, 121 - 141, ISBN/ISSN: 1-880611-84-8.

Birgersson, L., Jouve, J., Jönsson, E., Asker, N., Andreasson, F., Golovko, O., Ahrens, L., & Sturve, J. (2021). Thyroid function and immune status in perch (*Perca fluviatilis*) from lakes contaminated with PFASs or PCBs. *Ecotoxicology and Environmental Safety*, *222*, 112495. https://doi.org/10.1016/j.ecoenv.2021.112495

Blanton, M. L., & Specker, J. L. (2007). The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction. *Critical Reviews in Toxicology*, *37*(1-2), 97–115. https://doi.org/10.1080/10408440601123529

Brande-Lavridsen, N., Korsgaard, B., Dahllöf, I., Strand, J., Tairova, Z., & Bjerregaard, P. (2013). Abnormalities in eelpout Zoarces viviparus upon chemical exposure. *Marine Environmental Research*, *92*, 87–94. https://doi.org/10.1016/j.marenvres.2013.09.004

Brar, N. K., Waggoner, C., Reyes, J. A., Fairey, R., & Kelley, K. M. (2010). Evidence for thyroid endocrine disruption in wild fish in San Francisco Bay, California, USA. Relationships to contaminant exposures. *Aquatic Toxicology (Amsterdam, Netherlands)*, *96*(3), 203–215.

https://doi.org/10.1016/j.aquatox.2009.10.023
Brette, F., Cros, C., Machado, B., Incardona, J. P., Scholz, N. L., & Block, B. A. (2014). Crude oil impairs cardiac excitation-contraction coupling in fish. *Biophysical Journal*, *106*(2), 732a. https://doi.org/10.1016/j.bpj.2013.11.4037
Briaudeau, T., Alves Dos Santos, L. A., Zorita, I., Izagirre, U., & Marigómez, I. (2021). Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles by waterborne exposure to benzo[a]pyrene. *Marine Environmental Research*, *170*(105351), 105351. https://doi.org/10.1016/j.marenvres.2021.105351
Briaudeau, T., Zorita, I., Izagirre, U., & Marigómez, I. (2020). Biological responses and toxicopathic effects elicited in *Solea senegalensis* juveniles on exposure to contaminated sediments under laboratory conditions. *The Science of the Total Environment*, *731*(138849), 138849. https://doi.org/10.1016/j.scitotenv.2020.138849
Broeg, K., Zander, S., Diamant, A., Körting, W., Krüner, G., Paperna, I., & Westernhagen, H. v. (1999). The use of fish metabolic, pathological and parasitological indices in pollution monitoring. *Helgoland Marine Research*, *53*(3-4), 171–194. https://doi.org/10.1007/s101520050023
Brooks, S., Harman, C., Zaldibar, B., Izagirre, U., Glette, T., & Marigómez, I. (2011). Integrated biomarker assessment of the effects exerted by treated produced water from an onshore natural gas processing plant in the North Sea on the mussel *Mytilus edulis*. *Marine Pollution Bulletin*, *62*(2), 327–339. https://doi.org/10.1016/j.marpolbul.2010.10.007
Bucke, D., Vethaak, D., Lang, T., & Mellergaard, S. (1996). *Common diseases and parasites of fish in the North Atlantic: Training guide for identification*. ICES Techniques in Marine Environmental Science, Vol. 19. 27 pp. https://doi.org/10.17895/ices.pub.5045
Cajaraville, M. P., Olabarrieta, I., & Marigomez, I. (1996). *In vitro* activities in mussel hemocytes as biomarkers of environmental quality: a case study in the Abra Estuary (Biscay Bay). *Ecotoxicology and Environmental Safety*, *35*(3), 253–260. https://doi.org/10.1006/eesa.1996.0108
Castro, M., Santos, M. M., Monteiro, N. M., & Vieira, N. (2004). Measuring lysosomal stability as an effective tool for marine coastal environmental monitoring. *Marine Environmental Research*, *58*(2-5), 741–745. https://doi.org/10.1016/j.marenvres.2004.03.088
Chora, S., McDonagh, B., Sheehan, D., Starita-Geribaldi, M., Roméo, M., & Bebianno, M. J. (2010). Ubiquitination and carbonylation of proteins in the clam *Ruditapes decussatus*, exposed to nonylphenol using redox proteomics. *Chemosphere*, *81*(10), 1212–1217. https://doi.org/10.1016/j.chemosphere.2010.09.038
Ciacci, C., Canonico, B., Bilaničová, D., Fabbri, R., Cortese, K., Gallo, G., Marcomini, A., Pojana, G., & Canesi, L. (2012). Immunomodulation by different types of N-oxides in the hemocytes of the marine bivalve *Mytilus galloprovincialis*. *PloS One*, *7*(5), e36937. https://doi.org/10.1371/journal.pone.0036937
Clarke, D. J., Burchell, B., & George, S. G. (1992). Differential expression and induction of UDP-glucuronosyltransferase isoforms in hepatic and extrahepatic tissues of a fish, *Pleuronectes platessa*: immunochemical and functional characterization. *Toxicology and Applied Pharmacology*, *115*(1), 130–136. https://doi.org/10.1016/0041-008x(92)90376-4
CORESET. (2013). HELCOM core indicators: Final report HELCOM CORESET project. *Balt. Sea Environ. Proc*, *136*.
Costa, P. M., Carreira, S., Costa, M. H., & Caeiro, S. (2013). Development of histopathological indices in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality. *Aquatic Toxicology (Amsterdam, Netherlands)*, *126*, 442–454. https://doi.org/10.1016/j.aquatox.2012.08.013
Costa, P. M., Neuparth, T. S., Caeiro, S., Lobo, J., Martins, M., Ferreira, A. M., Caetano, M., Vale, C., DelValls, T. A., & Costa, M. H. (2011). Assessment of the genotoxic potential of contaminated estuarine sediments in fish peripheral blood: laboratory versus in situ studies. *Environmental Research*, *111*(1), 25–36. https://doi.org/10.1016/j.envres.2010.09.011
Courant, F., Arpin-Pont, L., Bonnefille, B., Vacher, S., Picot-Groz, M., Gomez, E., & Fenet, H. (2018). Exposure of marine mussels to diclofenac: modulation of prostaglandin biosynthesis. *Environmental Science and Pollution Research International*, *25*(7), 6087–6094.

https://doi.org/10.1007/s11356-017-9228-6
Dailianis, S., Domouhtsidou, G. P., Raftopoulou, E., Kaloyianni, 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*(4), 443–470. https://doi.org/10.1016/S0141-1136(03)00005-9
Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., & Colombo, R. (2003). Protein carbonyl groups as biomarkers of oxidative stress. *Clinica Chimica Acta; International Journal of Clinical Chemistry*, *329*(1-2), 23–38. https://doi.org/10.1016/s0009-8981(03)00003-2
Davies, I. M. and Vethaak, A. D. (Ed.). (2012). *Integrated marine environmental monitoring of chemicals and their effects*. ICES Cooperative Research Report. Vol 315. 289 pp. https://doi.org/10.17895/ices.pub.5403
Deal, C. K., & Volkoff, H. (2020). The Role of the Thyroid Axis in Fish. *Frontiers in Endocrinology*, *11*, 596585. https://doi.org/10.3389/fendo.2020.596585
Dimitriadis, V. K., Domouhtsidou, G. P., & Cajaraville, M. P. (2004). Cytochemical and histochemical aspects of the digestive gland cells of the mussel *Mytilus galloprovincialis* (L.) in relation to function. *Journal of Molecular Histology*, *35*(5), 501–509. https://doi.org/10.1023/b:hijo.0000045952.87268.76
Dorts, J., Silvestre, F., Tu, H. T., Tyberghein, A.-E., Phuong, N. T., & Kestemont, P. (2009). Oxidative stress, protein carbonylation and heat shock proteins in the black tiger shrimp, *Penaeus monodon*, following exposure to endosulfan and deltamethrin. *Environmental Toxicology and Pharmacology*, *28*(2), 302–310. https://doi.org/10.1016/j.etap.2009.05.006
Draper, H. H., & Hadley, M. (1990). Malondialdehyde determination as index of lipid peroxidation. *Methods in Enzymology*, *186*, 421–431. https://doi.org/10.1016/0076-6879(90)86135-i
Einsporn, S., Broeg, K., & Koehler, A. (2005). The Elbe flood 2002 - toxic effects of transported contaminants in flatfish and mussels of the Wadden Sea. *Marine Pollution Bulletin*, *50*(4), 423–429. https://doi.org/10.1016/j.marpolbul.2004.11.027
Faggio, C., Tsarpali, V., & Dailianis, S. (2018). Mussel digestive gland as a model tissue for assessing xenobiotics: An overview. *The Science of the Total Environment*, *636*, 220–229. https://doi.org/10.1016/j.scitotenv.2018.04.264
Fang, C., Huang, Q., Ye, T., Chen, Y., Liu, L., Kang, M., Lin, Y., Shen, H., & Dong, S. (2013). Embryonic exposure to PFOS induces immunosuppression in the fish larvae of marine medaka. *Ecotoxicology and Environmental Safety*, *92*, 104–111. https://doi.org/10.1016/j.ecoenv.2013.03.005
Fernandes, D., Bebianno, M. J., & Porte, C. (2008). Hepatic levels of metal and metallothioneins in two commercial fish species of the Northern Iberian shelf. *The Science of the Total Environment*, *391*(1), 159–167. https://doi.org/10.1016/j.scitotenv.2007.10.057
Frommel, A. Y., Maneja, R., Lowe, D., Malzahn, A. M., Geffen, A. J., Folkvord, A., Piatkowski, U., Reusch, T. B. H., & Clemmesen, C. (2012). Severe tissue damage in Atlantic cod larvae under increasing ocean acidification. *Nature Climate Change*, *2*(1), 42–46. https://doi.org/10.1038/nclimate1324
Gao, Y., Xu, H., Li, L., & Niu, C. (2020). Immune defense parameters of wild fish as sensitive biomarkers for ecological risk assessment in shallow sea ecosystems: A case study with wild mullet (*Liza haematocheila*) in Liaodong Bay. *Ecotoxicology and Environmental Safety*, *194*(110337), 110337. https://doi.org/10.1016/j.ecoenv.2020.110337
George, S. G. (2009). Enzymology and molecular biology of phase II xenobiotic-conjugating enzymes in fish. *Aquatic Toxicology*, *4*, 37–85.
Gillis, P. L., Higgins, S. K., & Jorge, M. B. (2014). Evidence of oxidative stress in wild freshwater mussels (*Lasmigona costata*) exposed to urban-derived contaminants. *Ecotoxicology and Environmental Safety*, *102*, 62–69. https://doi.org/10.1016/j.ecoenv.2013.12.026
Grundy, M. M., Moore, M. N., Howell, S. M., & Ratcliffe, N. A. (1996a). Phagocytic reduction and effects on lysosomal membranes by polycyclic aromatic hydrocarbons, in haemocytes of *Mytilus edulis*. *Aquatic Toxicology (Amsterdam, Netherlands)*, *34*(4), 273–290. https://doi.org/10.1016/0166-445x(95)00044-5
Grundy, M. M., Ratcliffe, N. A., & Moore, M. N. (1996b). Immune inhibition in marine mussels by

polycyclic aromatic hydrocarbons. *Marine Environmental Research*, *42*(1-4), 187–190. https://doi.org/10.1016/0141-1136(95)00033-x

Han, Z., Liu, Y., Wu, D., Zhu, Z., & Lü, C. (2012). Immunotoxicity and hepatotoxicity of PFOS and PFOA in tilapia (*Oreochromis niloticus*). *Chinese Journal of Geochemistry*, *31*(4), 424–430. https://doi.org/10.1007/s11631-012-0593-z

Hedman, J. E., Rüdel, H., Gercken, J., Bergek, S., Strand, J., Quack, M., Appelberg, M., Förlin, L., Tuvikene, A., & Bignert, A. (2011). Eelpout (*Zoarces viviparus*) in marine environmental monitoring. *Marine Pollution Bulletin*, *62*(10), 2015–2029. https://doi.org/10.1016/j.marpolbul.2011.06.028

Huang, M., Jiao, J., Wang, J., Xia, Z., & Zhang, Y. (2018). Exposure to acrylamide induces cardiac developmental toxicity in zebrafish during cardiogenesis. *Environmental Pollution (Barking, Essex: 1987)*, *234*, 656–666. https://doi.org/10.1016/j.envpol.2017.11.095

Hylland, K. 2004. Biological effects of contaminants: Quantification of δ-aminolevulinic acid dehydratase (ALA-D) activity in fish blood. ICES Techniques in Marine Environmental Sciences, No. 34. 9 pp.

Hylland, K., Bellas, J., Giltrap, M., & Brooks, S. (2021). *ICES Viewpoint background document: How can we quantify and manage the impact of chemical pollution in the oceans? (Ad Hoc)* (No. 102; Vol. 3). ICES Scientific Reports. https://doi.org/10.17895/ices.pub.5446

Hylland, K., Ruus, A., Grung, M., & Green, N. (2009). Relationships between physiology, tissue contaminants, and biomarker responses in Atlantic cod (Gadus morhua L.). *Journal of Toxicology and Environmental Health. Part A*, *72*(3-4), 226–233. https://doi.org/10.1080/15287390802539129

Hylland, K., Skei, B. B., Brunborg, G., Lang, T., Gubbins, M. J., le Goff, J., & Burgeot, T. (2017). DNA damage in dab (Limanda limanda) and haddock (*Melanogrammus aeglefinus*) from European seas. *Marine Environmental Research*, *124*, 54–60. https://doi.org/10.1016/j.marenvres.2016.01.001

ICES. (2012). *Working Group on Pathology and Diseases of Marine Organisms (WGPDMO)*. ICES Scientific Reports. 1:62. 35 pp. https://doi.org/10.17895/ices.pub.5603

Incardona, J., Carls, M., Holland, L., Linbo, T. L., Baldwin, D., Myers, M., Peck, K. A., Tagal, M., Rice, S., & Scholz, N. (2015). Very low embryonic crude oil exposures cause lasting cardiac defects in salmon and herring. *Scientific Reports*, *5*. https://doi.org/10.1038/srep13499

Incardona, J. P., Linbo, T. L., French, B. L., Cameron, J., Peck, K. A., Laetz, C. A., Hicks, M. B., Hutchinson, G., Allan, S. E., Boyd, D. T., Ylitalo, G. M., & Scholz, N. L. (2021). Low-level embryonic crude oil exposure disrupts ventricular ballooning and subsequent trabeculation in Pacific herring. *Aquatic Toxicology (Amsterdam, Netherlands)*, *235*(105810), 105810. https://doi.org/10.1016/j.aquatox.2021.105810

Izagirre, U., Ramos, R., & Marigómez, I. (2008). Natural variability in size and membrane stability of lysosomes in mussel digestive cells: seasonal and tidal zonation. *Marine Ecology Progress Series*, *372*, 105–117. https://doi.org/10.3354/MEPS07656

Izagirre, U. (2008). *Contribution to the interpretation of lysosomal biomarkers in marine organisms based on the mechanistic understanding of the lysosomal responses to pollutants* [PhD Thesis]. Univ Basque Country. 306pp.

Jacobsson, A., Neuman, E., & Thoresson, G. (1986). The viviparous blenny as an indicator of environmental effects of harmful substances. *Ambio*, *15*(4), 236–238.

Joshy, A., Sharma, S. R. K., Mini, K. G., Gangadharan, S., & Pranav, P. (2022). Histopathological evaluation of bivalves from the southwest coast of India as an indicator of environmental quality. *Aquatic Toxicology (Amsterdam, Netherlands)*, *243*(106076), 106076. https://doi.org/10.1016/j.aquatox.2022.106076

Kishi, S. (2004). Functional aging and gradual senescence in zebrafish. *Annals of the New York Academy of Sciences*, *1019*(1), 521–526. https://doi.org/10.1196/annals.1297.097

Knapen, D., Stinckens, E., Cavallin, J. E., Ankley, G. T., Holbech, H., Villeneuve, D. L., & Vergauwen, L. (2020). Toward an AOP network-based tiered testing strategy for the assessment of thyroid hormone disruption. *Environmental Science & Technology*, *54*(14), 8491–8499. https://doi.org/10.1021/acs.est.9b07205

Köhler, A. (1991). Lysosomal perturbations in fish liver as indicators for toxic effects of

environmental pollution. *Comparative Biochemistry and Physiology. C, Comparative Pharmacology and Toxicology*, *100*(1-2), 123–127. https://doi.org/10.1016/0742-8413(91)90137-i

Köhler, A., Deisemann, H., & Lauritzen, B. (1992). Histological and cytochemical indices of toxic injury in the liver of dab Limanda limanda. *Marine Ecology Progress Series. Oldendorf*, *91*(1), 141–153. https://www.int-res.com/articles/meps/91/m091p141.pdf

Köhler, A., Wahl, E., & Söffker, K. (2002). Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish ( *Platichthys flesus* (L.)). *Environmental Toxicology and Chemistry*, *21*(11), 2434–2444. https://doi.org/10.1002/etc.5620211124

Kopecka, J., Lehtonen, K. K., Barsiene, J., Broeg, K., Vuorinen, P. J., Gercken, J., & 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*(8-9), 406–421. https://doi.org/10.1016/j.marpolbul.2006.03.008

Kroon, F., Streten, C., & Harries, S. (2017). A protocol for identifying suitable biomarkers to assess fish health: A systematic review. *PloS One*, *12*(4), e0174762. https://doi.org/10.1371/journal.pone.0174762

Kwok, C.-T., van de Merwe, J. P., Chiu, J. M. Y., & Wu, R. S. S. (2012). Antioxidant responses and lipid peroxidation in gills and hepatopancreas of the mussel Perna viridis upon exposure to the red-tide organism Chattonella marina and hydrogen peroxide. *Harmful Algae*, *13*, 40–46. https://doi.org/10.1016/j.hal.2011.10.001

Lang, T., & Wosniok, W. (2008). The Fish Disease Index: a method to assess wild fish disease data in the context of marine environmental monitoring. *2008 Annual Science Conference, Halifax, Canada. CM 2008/D:01*. https://doi.org/10.17895/ices.pub.25243567

Larsson, D. G. J., & Förlin, L. (2002). Male-biased sex ratios of fish embryos near a pulp mill: temporary recovery after a short-term shutdown. *Environmental Health Perspectives*, *110*(8), 739–742. https://doi.org/10.1289/ehp.02110739

Lastumäki, A., Turja, R., Brenner, M., Vanninen, P., Niemikoski, H., Butrimavičienė, L., Stankevičiūtė, M., & Lehtonen, K. K. (2020). Biological effects of dumped chemical weapons in the Baltic Sea: A multi-biomarker study using caged mussels at the Bornholm main dumping site. *Marine Environmental Research*, *161*, 105036. https://doi.org/10.1016/j.marenvres.2020.105036

Le, T. T. Y., Zimmermann, S., & Sures, B. (2016). How does the metallothionein induction in bivalves meet the criteria for biomarkers of metal exposure? *Environmental Pollution (Barking, Essex: 1987)*, *212*, 257–268. https://doi.org/10.1016/j.envpol.2016.01.070

Levine, R. L., Wehr, N., Williams, J. A., Stadtman, E. R., & Shacter, E. (2000). Determination of carbonyl groups in oxidized proteins. *Methods in Molecular Biology (Clifton, N.J.)*, *99*, 15–24. https://doi.org/10.1385/1-59259-054-3:15

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

Lowe, D.M., Soverchia, C., and Moore, M.N. (1995b). Lysosomal membrane responses in the blood and digestive cells of mussels experimentally exposed to fluoranthene. Aquat Toxicol 33: 105–112.

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*, 411–414.

Marris, C. R., Kompella, S. N., Miller, M. R., Incardona, J. P., Brette, F., Hancox, J. C., Sørhus, E., & Shiels, H. A. (2020). Polyaromatic hydrocarbons in pollution: a heart-breaking matter. *The Journal of Physiology*, *598*(2), 227–247. https://doi.org/10.1113/JP278885

Martínez-Gómez, C., Benedicto, J., Campillo, J. A., & Moore, M. (2008). Application and evaluation of the neutral red retention (NRR) assay for lysosomal stability in mussel populations along the Iberian Mediterranean coast. *Journal of Environmental Monitoring: JEM*, *10*(4), 490–499. https://doi.org/10.1039/b800441m

Martínez-Gómez, C., Bignell, J., & Lowe, D. (2015). Lysosomal membrane stability in mussels. *ICES Techniques in Marine Environmental Sciences No. 56. 41 Pp*. https://doi.org/10.17895/ices.pub.5084

Martínez-Gómez, C., Fernández, B., Benedicto, J., Valdés, J., Campillo, J. A., León, V. M., &

Vethaak, A. D. (2012). Health status of red mullets from polluted areas of the Spanish Mediterranean coast, with special reference to Portmán (SE Spain). *Marine Environmental Research*, *77*, 50–59. https://doi.org/10.1016/j.marenvres.2012.02.002

Martínez-Gómez, C., Fernández, B., Robinson, C. D., Campillo, J. A., León, V. M., Benedicto, J., Hylland, K., & Vethaak, A. D. (2017). Assessing environmental quality status by integrating chemical and biological effect data: The Cartagena coastal zone as a case. *Marine Environmental Research*, *124*, 106–117. https://doi.org/10.1016/j.marenvres.2016.04.008

Martínez-Gómez, C., & Vethaak, A. D. (2019). Understanding the impact of chemicals on marine fish populations: the need for an integrative approach involving population and disease ecology. *Current Opinion in Environmental Science & Health*, *11*, 71–77. https://doi.org/10.1016/j.coesh.2019.08.001

Martyniuk, V., Gylytė, B., Matskiv, T., Khoma, V., Tulaidan, H., Gnatyshyna, L., Orlova-Hudim, K., Manusadžianas, L., & Stoliar, O. (2022). Stress responses of bivalve mollusc Unio tumidus from two areas to ibuprofen, microplastic and their mixture. *Ecotoxicology (London, England)*, *31*(9), 1369–1381. https://doi.org/10.1007/s10646-022-02594-8

Mellergaard, S., & Nielsen, E. (1995). Impact of oxygen deficiency on the disease status of common dab Limanda limanda. *Diseases of Aquatic Organisms*, *22*(2), 101–114. https://doi.org/10.3354/dao022101

Moore, M., Lowe, D., & Köhler, A. (2004). Measuring lysosomal membrane stability. ICES Techniques in Marine Environmental Sciences. *ICES Techniques in Marine Environmental Sciences*, *36*, 1–31. https://doi.org/10.17895/ices.pub.5060

Morthorst, J. E., Brande-lavridsen, N., Korsgaard, B., & Bjerregaard, P. (2014). 17 β -Estradiol Causes Abnormal Development in Embryos of the Viviparous Eelpout. *Environmental Science & Technology*, *48*, 14668−14676. https://doi.org/dx.doi.org/10.1021/es5046698

Mullur, R., Liu, Y.-Y., & Brent, G. A. (2014). Thyroid hormone regulation of metabolism. *Physiological Reviews*, *94*(2), 355–382. https://doi.org/10.1152/physrev.00030.2013

Neuman, E., Sandström, O., & Thoresson, G. (1999). Guidelines for coastal fish monitoring. *National Board of Fisheries, Institute of Coastal Research, Öregrund, Sweden. 44 Pp*.

Olafson, K. W., & Thompson, J. A. J. (1974). Isolation of heavy metal binding proteins from marine vertebrates. *Marine Biology*, *28*(2), 83–86. https://doi.org/10.1007/bf00396298

Oliva, M., José Vicente, J., Gravato, C., Guilhermino, L., & Dolores Galindo-Riaño, M. (2012). Oxidative stress biomarkers in Senegal sole, Solea senegalensis, to assess the impact of heavy metal pollution in a Huelva estuary (SW Spain): seasonal and spatial variation. *Ecotoxicology and Environmental Safety*, *75*(1), 151–162. https://doi.org/10.1016/j.ecoenv.2011.08.017

Omar, W. A., Saleh, Y. S., & Marie, M.-A. S. (2016). The use of biotic and abiotic components of Red Sea coastal areas as indicators of ecosystem health. *Ecotoxicology (London, England)*, *25*(2), 253–266. https://doi.org/10.1007/s10646-015-1584-8

OSPAR. (2013). Technical Annex on Supporting Parameters for Biological Effects Measurements in Fish and Mussels. Background Document and Technical Annexes for Biological Effects Monitoring, Update 2013. *978-1-909159-22-8 (2013), Pp. 211-219 Monitoring and Assessment Series 589/2013*.

Parrino, V., Costa, G., Cannavà, C., Fazio, E., Bonsignore, M., Concetta, S., Piccione, G., & Fazio, F. (2019). Flow cytometry and micro-Raman spectroscopy: Identification of hemocyte populations in the mussel Mytilus galloprovincialis (Bivalvia: Mytilidae) from Faro Lake and Tyrrhenian Sea (Sicily, Italy). *Fish & Shellfish Immunology*, *87*, 1–8. https://doi.org/10.1016/j.fsi.2018.12.067

Pedersen, A. B., & Babayan, S. A. (2011). Wild immunology: Wild immunology. *Molecular Ecology*, *20*(5), 872–880. https://doi.org/10.1111/j.1365-294X.2010.04938.x

Pereira, P., de Pablo, H., Vale, C., & Pacheco, M. (2010). Combined use of environmental data and biomarkers in fish (Liza aurata) inhabiting a eutrophic and metal-contaminated coastal system - Gills reflect environmental contamination. *Marine Environmental Research*, *69*(2), 53–62. https://doi.org/10.1016/j.marenvres.2009.08.003

Piva, F., Ciaprini, F., Onorati, F., Benedetti, M., Fattorini, D., Ausili, A., & Regoli, F. (2011). Assessing sediment hazard through a weight of evidence approach with bioindicator organisms: a practical model to elaborate data from sediment chemistry, bioavailability, biomarkers and ecotoxicological bioassays. *Chemosphere*, *83*(4), 475–485.

https://doi.org/10.1016/j.chemosphere.2010.12.064
Power, D. M., Llewellyn, L., Faustino, M., Nowell, M. A., Björnsson, B. T., Einarsdottir, I. E., Canario, A. V., & Sweeney, G. E. (2001). Thyroid hormones in growth and development of fish. *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *130*(4), 447–459. https://www.ncbi.nlm.nih.gov/pubmed/11738632
Rehberger, K., Werner, I., Hitzfeld, B., Segner, H., & Baumann, L. (2017). 20 Years of fish immunotoxicology – what we know and where we are. *Critical Reviews in Toxicology*, *47*(6), 516–542. https://doi.org/10.1080/10408444.2017.1288024
Reichert, W. L., French, B. L., & Stein, J. E. (1999). *Biological effects of contaminants: Measurement of DNA adducts in fish by 32P-postlabelling*. International Council for the Exploration of the Sea (ICES). https://doi.org/10.25607/OBP-274
Renault, T. (2015). Immunotoxicological effects of environmental contaminants on marine bivalves. *Fish & Shellfish Immunology*, *46*(1), 88–93. https://doi.org/10.1016/j.fsi.2015.04.011
Renwrantz, L., Siegmund, E., & Woldmann, M. (2013). Variations in hemocyte counts in the mussel, Mytilus edulis: similar reaction patterns occur in disappearance and return of molluscan hemocytes and vertebrate leukocytes. *Comparative Biochemistry and Physiology. Part A, Molecular & Integrative Physiology*, *164*(4), 629–637. https://doi.org/10.1016/j.cbpa.2013.01.021
Repetto, G., del Peso, A., & Zurita, J. L. (2008). Neutral red uptake assay for the estimation of cell viability/cytotoxicity. *Nature Protocols*, *3*(7), 1125–1131. https://doi.org/10.1038/nprot.2008.75
Reznick, A., & Packer, L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods in Enzymology*, *233*, 357–363. https://doi.org/10.1016/S0076-6879(94)33041-7
Schiedek, D., Broeg, K., Barsiene, J., Lehtonen, K. K., Gercken, J., Pfeifer, S., Vuontisjärvi, H., Vuorinen, P. J., Dedonyte, 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*(8-9), 387–405. https://doi.org/10.1016/j.marpolbul.2005.11.013
Schlenk, D., Goldstone, J., James, M. O., & van den Hurk, P. (2008). Biotransformation in Fishes 1. In *Toxicology of fishes* (pp. 60–120). CRC press. https://doi.org/10.1201/9781003160694-3/biotransformation-fishes-1-daniel-schlenk-jed-goldstone-margaret-james-peter-van-den-hurk
Seglen, P. O. (1983). [59] Inhibitors of lysosomal function. *Methods in Enzymology*. https://www.sciencedirect.com/science/article/pii/S0076687983960639
Segner, H., Bailey, C., Tafalla, C., & Bo, J. (2021). Immunotoxicity of xenobiotics in fish: A role for the aryl hydrocarbon receptor (AhR)? *International Journal of Molecular Sciences*, *22*(17), 9460. https://doi.org/10.3390/ijms22179460
Segner, H., Wenger, M., Möller, A. M., Köllner, B., & Casanova-Nakayama, A. (2012). Immunotoxic effects of environmental toxicants in fish—how to assess them? Environmental Science and Pollution Research, 19(7), 2465–2476.
Shacter, E., Williams, J. A., Lim, M., & Levine, R. L. (1994). Differential susceptibility of plasma proteins to oxidative modification: examination by western blot immunoassay. *Free Radical Biology & Medicine*, *17*(5), 429–437. https://doi.org/10.1016/0891-5849(94)90169-4
Shi, W., Han, Y., Guo, C., Zhao, X., Liu, S., Su, W., Zha, S., Wang, Y., & Liu, G. (2017). Immunotoxicity of nanoparticle nTiO2 to a commercial marine bivalve species, Tegillarca granosa. *Fish & Shellfish Immunology*, *66*, 300–306. https://doi.org/10.1016/j.fsi.2017.05.036
Siscar, R., Koenig, S., Torreblanca, A., & Solé, M. (2014). The role of metallothionein and selenium in metal detoxification in the liver of deep-sea fish from the NW Mediterranean Sea. *The Science of the Total Environment*, *466-467*, 898–905. https://doi.org/10.1016/j.scitotenv.2013.07.081
Sørhus, E., Nakken, C. L., Donald, C. E., Ripley, D. M., Shiels, H. A., & Meier, S. (2023). Cardiac toxicity of phenanthrene depends on developmental stage in Atlantic cod (*Gadus morhua*). *The Science of the Total Environment*, *881*(163484), 163484. https://doi.org/10.1016/j.scitotenv.2023.163484
Soto, M., Zaldibar, B., Cancio, I., Taylor, M. G., Turner, M., Morgan, A. J., & 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. *The Histochemical*

*Journal*, *34*(6-7), 273–280. https://doi.org/10.1023/a:1023322423654
Srikanth, K., Pereira, E., Duarte, A. C., & Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish--a review. *Environmental Science and Pollution Research International*, *20*(4), 2133–2149. https://doi.org/10.1007/s11356-012-1459-y
Stadtman, E. R., & Levine, R. L. (2000). Protein oxidation. *Annals of the New York Academy of Sciences*, *899*(1), 191–208. https://doi.org/10.1111/j.1749-6632.2000.tb06187.x
Stentiford, G. D., Bignell, J. P., Lyons, B. P., Thain, J. E., & 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. https://doi.org/10.3354/meps08693
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*(2004), 37–46.
Strand, J., & Dahllöf, I. (2005). *Teknisk anvisning for marin overvågning. 4.5 Biologisk effektmonitering-muslinger: NOVANA*.
Sturve, J., Berglund, A., Balk, L., Broeg, K., Böhmert, B., Massey, S., Savva, D., Parkkonen, J., Stephensen, E., Koehler, A., & Förlin, L. (2005). Effects of dredging in Göteborg harbor, Sweden, assessed by biomarkers in eelpout (Zoarces viviparus). *Environmental Toxicology and Chemistry / SETAC*, *24*(8), 1951–1961. https://www.ncbi.nlm.nih.gov/pubmed/16152967
Sueiro, M. C., Awruch, C., Gilardoni, C., Demetrio, M., & Palacios, M. G. (2020). Immunity and health of two wild marine fishes naturally exposed to anthropogenic pollution. *The Science of the Total Environment*, *726*(138303), 138303. https://doi.org/10.1016/j.scitotenv.2020.138303
Sundberg, H., Hanson, M., Liewenborg, B., Zebühr, Y., Broman, D., & Balk, L. (2007). Dredging associated effects: maternally transferred pollutants and DNA adducts in feral fish. *Environmental Science & Technology*, *41*(8), 2972–2977. https://doi.org/10.1021/es070073j
Townsend, D. M., & Tew, K. D. (2003). The role of glutathione-S-transferase in anti-cancer drug resistance. *Oncogene*, *22*(47), 7369–7375. https://doi.org/10.1038/sj.onc.1206940
Tairova, Z. M., Strand, J., Chevalier, J., & Andersen, O. (2012). PAH biomarkers in common eelpout (Zoarces viviparus) from Danish waters. *Marine Environmental Research*, *75*, 45–53. https://doi.org/10.1016/j.marenvres.2011.09.005
Turja, R., Benito, D., Ahvo, A., Izagirre, U., Lekube, X., Stankevičiūtė, M., Butrimavičienė, L., Soto, M., & Lehtonen, K. K. (2023). Biomarker responses in mussels (Mytilus trossulus) from the Baltic Sea exposed to water-accommodated fraction of crude oil and a dispersant at different salinities. *Marine Pollution Bulletin*, *192*, 115100. https://doi.org/10.1016/j.marpolbul.2023.115100
Turja, R., Soirinsuo, A., Budzinski, H., Devier, M. H., & Lehtonen, K. K. (2013). Biomarker responses and accumulation of hazardous substances in mussels (*Mytilus trossulus*) transplanted along a pollution gradient close to an oil terminal in the Gulf of Finland (Baltic Sea). *Comparative Biochemistry and Physiology. Toxicology & Pharmacology: CBP*, *157*(1), 80–92. https://doi.org/10.1016/j.cbpc.2012.09.006
Tysklind, N., Taylor, M. I., Lyons, B. P., Goodsir, F., McCarthy, I. D., & Carvalho, G. R. (2013). Population genetics provides new insights into biomarker prevalence in dab (Limanda limanda L.): a key marine biomonitoring species. *Evolutionary Applications*, *6*(6), 891–909. https://doi.org/10.1111/eva.12074
Varanasi, U., & Stein, J. E. (1991). Disposition of xenobiotic chemicals and metabolites in marine organisms. *Environmental Health Perspectives*, *90*, 93–100. https://doi.org/10.1289/ehp.90-1519508
Vethaak, A., Bucke, D., Lang, T., Wester, P., Jol, J., & Carr, M. (1992). Fish disease monitoring along A pollution transect - A case-study using dab *Limanda-Limanda* in the German bight. *Marine Ecology Progress Series*, *91*(1/3), 173–192. https://doi.org/10.3354/MEPS091173
Vethaak, A. D., & ap Rheinallt, T. (1992). Fish disease as a monitor for marine pollution: the case of the North Sea. *Reviews in Fish Biology and Fisheries*, *2*(1), 1–32. https://doi.org/10.1007/bf00042915
Vethaak, A. D., Jol, J. G., Meijboom, A., Eggens, M. L., Rheinallt, T., Wester, P. W., van de Zande, T., Bergman, A., Dankers, N., Ariese, F., Baan, R. A., Everts, J. M., Opperhuizen, A., & Marquenie,

J. M. (1996). Skin and liver diseases induced in flounder (Platichthys flesus) after long-term exposure to contaminated sediments in large-scale mesocosms. *Environmental Health Perspectives*, *104*(11), 1218–1229. https://doi.org/10.1289/ehp.961041218

Vethaak, A., & Jol, J. (1996). Diseases of flounder Platichthys flesus in Dutch coastal and estuarine waters, with particular reference to environmental stress factors. I. Epizootiology of gross lesions. *Diseases of Aquatic Organisms*, *26*, 81–97. https://doi.org/10.3354/DAO026081

Viarengo, A., Ponzano, E., Dondero, F., & Fabbri, R. (1997). A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. *Marine Environmental Research*, *44*(1), 69–84. https://doi.org/10.1016/s0141-1136(96)00103-1

Vieira, C. E. D., Marques, J. A., da Silva, N. G., Bevitório, L. Z., Zebral, Y. D., Maraschi, A. C., Costa, S. R., Costa, P. G., Damasceno, E. M., Pirovani, J. C. M., do Vale-Oliveira, M., Souza, M. M., de Martinez Gaspar Martins, C., Bianchini, A., & Sandrini, J. Z. (2022). Ecotoxicological impacts of the Fundão dam failure in freshwater fish community: Metal bioaccumulation, biochemical, genetic and histopathological effects. *The Science of the Total Environment*, *832*(154878), 154878. https://doi.org/10.1016/j.scitotenv.2022.154878

Widdows, J., & Staff, Fred. (2006). Biological effects of contaminants: measurement of scope for growth in mussels. International Council for the Exploration of the Sea (ICES). https://doi.org/10.25607/OBP-224

Williams, G. R. (2008). Neurodevelopmental and neurophysiological actions of thyroid hormone. *Journal of Neuroendocrinology*, *20*(6), 784–794. https://doi.org/10.1111/j.1365-2826.2008.01733.x

Winterbourn, C., & Buss, I. H. (1999). Protein carbonyl measurement by enzyme-linked immunosorbent assay. *Methods in Enzymology*, *300*, 106–111. https://doi.org/10.1016/S0076-6879(99)00118-4

Winzer, K., Winston, G. W., Becker, W., Van Noorden, C. J., & Köehler, A. (2001). Sex-related responses to oxidative stress in primary cultured hepatocytes of European flounder (Platichthys flesus L.). *Aquatic Toxicology (Amsterdam, Netherlands)*, *52*(2), 143–155. https://doi.org/10.1016/s0166-445x(00)00137-5

Wood, C. M. (2011). Fish physiology: Homeostasis and toxicology of essential metals: Volume 31A. Academic Press. https://doi.org/10.1016/c2009-0-01969-2

Zhu, L.-Y., Nie, L., Zhu, G., Xiang, L.-X., & Shao, J.-Z. (2013). Advances in research of fish immune-relevant genes: a comparative overview of innate and adaptive immunity in teleosts. *Developmental and Comparative Immunology*, *39*(1-2), 39–62. https://doi.org/10.1016/j.dci.2012.04.001

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. *Environmental Pollution (Barking, Essex: 1987)*, *148*(1), 236–250. https://doi.org/10.1016/j.envpol.2006.10.022

# Annex B – the CHASE tool

The HELCOM Hazardous Substances Status Assessment Tool (CHASE) was developed in 2010 for the HELCOM Holistic Assessment (HOLAS), with the aim of implementing the Baltic Sea Action Plan and fulfilling the requirements of the Marine Strategy Framework Directive (MSFD) to assess the ‘good environmental status’ of marine waters. This indicator-based assessment tool integrates data on hazardous substances found in water, sediments, and biota, as well as biological effects indicators. For each substance or biological effect, a ratio is calculated between the observed concentration or response and its respective threshold value. The overall assessment follows the ‘one out, all out’ (OO-AO) principle (Andersen et al., 2010).

The tool first evaluates the four components—water, sediment, biota, and biological effects—separately. The final status is determined based on the lowest status among the four components (Fig. 1), following OO-AO principle. This approach is deemed suitable, as each component represents a distinct facet of contamination, and equal weight is given to all of them as they are considered potentially equally harmful to the ecosystem.

The integrated assessment assigns each assessment unit (i.e., spatial unit) into one of five status classes: bad, poor, moderate, good, and high. Status classifications of bad, poor, or moderate indicate an environmental state 'affected' by hazardous substances, while good or high statuses indicate an 'unaffected' state. This classification system is essentially binary—‘unaffected’ versus ‘affected’—distinguished by a threshold value: areas with a score of <1.0 are considered 'unaffected,' while those with a score of >1.0 are regarded as 'affected.'

For biological effects, CHASE approach integrates biomarkers by the sum of the ratios of measured responses to target values and then dividing by the root mean square of the number of indicators. This prevents the “dilution” of high-impact indicators (e.g., ratios larger than five) by low-impact ones. This integration produces more reliable results and is suited for dealing with geographical variability, compared with other tested metrics (Andersen *et al.*, 2016). The number of elements used in CHASE is also critical for the final assessment result. If one of the four components are missing in an assessment unit, the assessment is more likely to yield a positive status result. Including biological effects indicators through CHASE indirectly accounts for the so-called 'cocktail effects' (cumulative effects of multiple contaminants) in the final assessment (Andersen et al., 2016).

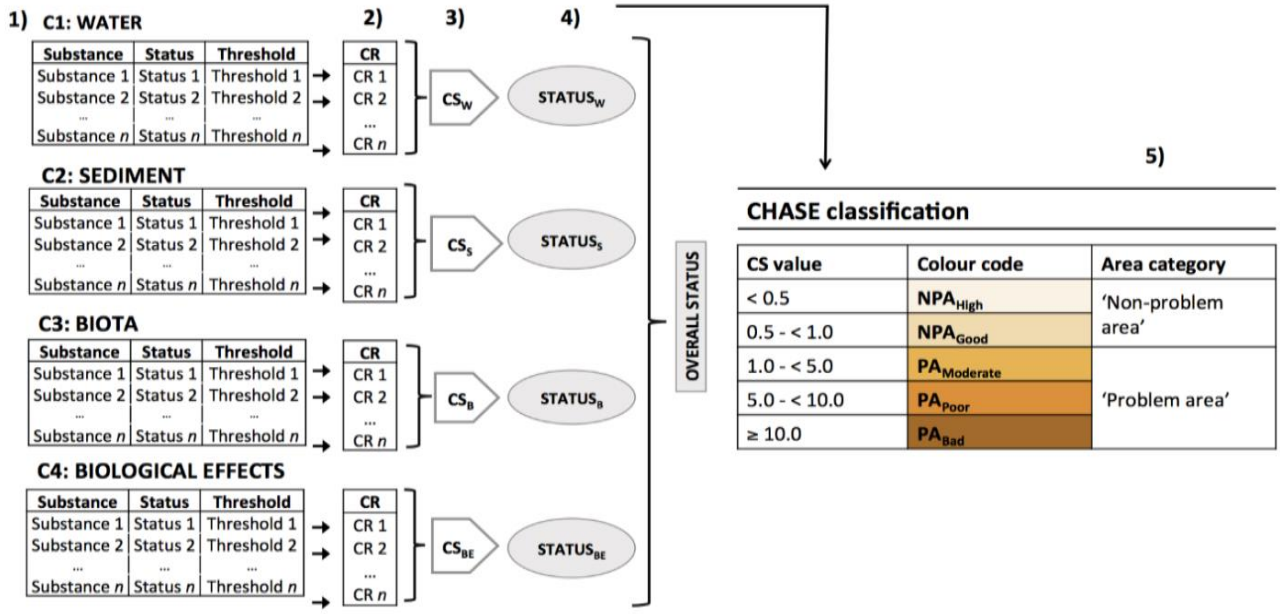


Figure 1. Schematic presentation of the CHASE tool. The four components (water, sediment, biota and biological effects) are filled with n indicators (Step 1) and their associated quantitative thresholds values from where the consequent Contamination Ratios (CR) are derived (Step 2). The Contamination Score (CS) (Step 3) are calculated to define the status class for each component (Step 4). The final status (Step 5) is determined by the 'one out, all out' principle, i.e. the worst status for any element determined the overall status for an assessment unit.

# Annex C – summary tables of protocols, QA and BAC

**Table C1**. Protocol, quality assurance and background assessment criteria (BAC) for recommended biological effect methods and relevant fish species. Note that sole, grey mullet and herring are also relevant fish species but there are no existing assessment criteria. Contrast: EU Horizon project 2024-2027 (CONTRAST Project).

| **Biomarker** | **Ref** | **Inter-calibration** | **Background Assessment Criteria** | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | dab | flounder | plaice | cod | eelpout | perch |
| Neoplasia or tumours | TIMES 38 | BEQUALM, 2013 | X | | | | | |
| Vitellogenin in plasma | TIMES 31 | Contrast, 2025 | | X | | X | | |
| Micronucleus frequency | TIMES 66 | Contrast, 2025 | X | X | | X | X | |
| DNA strand breaks in blood cells | TIMES 58 | | X | | | X | | |
| Cytochrome P4501A activity (EROD) in liver | TIMES 57 (23), 13 | BEQUALM 2022 Contrast, 2025 | X | X | X | X | X | X |
| AChE inhibition in muscle | TIMES 22 | Contrast, 2025 | X | X | | | | |

**Table C2**. Protocol, quality assurance and background assessment criteria (BAC) for recommended biological effect methods and relevant bivalve species. Note that *Macoma balthica* is also a relevant bivalve species but there are no existing assessment criteria.

| **Effect method (biomarker)** | **Ref** | **Inter-calibration** | **Background Assessment Criteria** | | | |
|---|---|---|---|---|---|---|
| | | | *Mytilus edulis* | *Mytilus trossulus* | *Mytilus galloprovincialis* | Any species |
| AChE activity in gill or foot | TIMES 22 | | X | | X | |
| Micronucleus frequency | TIMES 66 | BEQUALM 2019 | X | X | X | |
| Lysosomal membrane stability (LMS) in hepatopancreas | TIMES 56 | | | | | X |

**Table C3.** Protocol, quality assurance and background assessment criteria (BAC) for relevant amphipod species.

| Effect method (biomarker) | Ref | Inter-calibration | Background Assessment Criteria | | |
|---|---|---|---|---|---|
| | | | Monoporeia | Pontoporeia | Gammarids |
| embryo malformation | TIMES 41 HELCOM 2013 | HOLAS III (HELCOM) BEACON 2023-2024 (Interreg) | X | X | X |

**Table C4.** Protocol, quality assurance and background assessment criteria (BAC) for relevant gastropod species.

| Effect method (biomarker) | Ref | Inter-calibration | Background Assessment Criteria | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Nucella | Tritia | Peringia | Neptunea | Buccinum | Littorina |
| imposex/ intersex indicators | TIMES 24 TIMES 37 | QUASI-MEME | X | X | X | X | X | X |

**Table C5.** Protocol, quality assurance and background assessment criteria (BAC) for bile PAH metabolites in relevant fish species. Note that sole, plaice, grey mullet and herring are also relevant fish species but there is no existing assessment criteria.

| | Ref | Intercalibration | Background Assessment Criteria | | | | |
|---|---|---|---|---|---|---|---|
| | | | dab | flounder | cod | eelpout | perch |
| PAH-metabolite concentration in bile | TIMES 39 | BEQUALM 2019 Contrast 2025 | X | X | X | X | X |

## Literature

HELCOM (2023). Reproductive disorders: malformed embryos of amphipods. HELCOM supplementary indicator report; https://indicators.helcom.fi/indicator/reproductive-disorders/

TIMES 13: Galgani, F., and Payne, J.F. 1991. Biological effects of contaminants: Microplate method for measurement of ethoxyresoruim-O-deethylase (EROD) in fish. ICES Techniques in Marine Environmental Sciences, No. 13.

TIMES 22: Bocquene, G., and Galgani, F. 1998. Biological effects of contaminants: Cholinesterase inhibition by organophosphate and carbamate compounds. ICES Techniques in Marine Environmental Sciences, No. 22.

TIMES 23: Stagg, R., and McIntosh, A. 1998. Biological effects of contaminants: Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorometric measurement of EROD activity. ICES Techniques in Marine Environmental Sciences, No. 23.

TIMES 24: Gibbs, P.E. 1999. Biological effects of contaminants: Use of imposex in the

dogwhelk (Nucella lapillus) as a bioindicator of tributyltin pollution. ICES Techniques in Marine Environmental Sciences, No. 24. 29 pp.

TIMES 31: Scott, A.P., and Hylland, K. 2002. Biological effects of contaminants: Radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) techniques for the measurement of marine fish vitellogenins. ICES Techniques in Marine Environmental Sciences, No. 31. 21pp.

TIMES 37: J. Oehlmann. 2004. Biological effects of contaminants: Use of intersex in the periwinkle (*Littorina littorea*) as a biomarker of tributyltin pollution. By. ICES Techniques in Marine Environmental Sciences, No. 37. 22 pp

TIMES 39: Ariese, F., Beyer, J., Jonsson, G., Porte, C., & Krahn, M. M. (2005). Review of analytical methods for determining metabolites of polycyclic aromatic compounds (PACs) in fish bile.

TIMES 41: Sundelin, B., Eriksson Wiklund, A-K., and Ford, A. T. 2008. Biological effects of contaminants: the use of embryo aberrations in amphipod crustaceans for measuring effects of environmental stressors. ICES Techniques in Marine Environmental Sciences No. 41. 21 pp.

TIMES 57: Stagg, R.; McIntosh, A.; Gubbins, M. J. (2016). Determination of CYP1A-dependent mono-oxygenase activity in dab by fluorimetric measurement of EROD activity in S9 or microsomal liver fractions. ICES Techniques in Marine Environmental Science (TIMES).

TIMES 58: Bean, T. P.; Akcha, F. (2016). Biological effects of contaminants: Assessing DNA damage in marine species through single-cell alkeline gel electrophoresis (comet) assay. ICES Techniques in Marine Environmental Science (TIMES).

TIMES 56: Martínez-Gómez, C., Bignell, J. and Lowe, D. 2015. Lysosomal membrane stability in mussels. ICES Techniques in Marine Environmental Sciences No. 56. 41 pp

TIMES 66: Stankevičiūtė, Milda; Gomes, Tânia; González, Juan Antonio Campillo (2022). Nuclear abnormalities in mussel haemocytes and fish erythrocytes. ICES Techniques in Marine Environmental Science (TIMES). Report.

## Annex D - SGEFF members

| Name | Surname(s) | Country (of institute) |
|---|---|---|
| Gastón | Alurralde | Sweden: University of Stockholm & Finland: HELCOM |
| Hannah | Anderson | Scotland; MSS |
| Marta | Assuncao | UK; Cefas |
| Ieva | Bārda | Latvia; LHEI |
| Juan | Bellas | Spain; IEO, CSIC |
| John | Bignell | UK; Cefas |
| Steven | Brooks | Norway; NIVA |
| Rob | Fryer | Scotland; MSS |
| Michelle | Giltrap | Ireland; Technical University, Dublin |
| Elena | Gorokhova | Sweden; University of Stockholm |
| Ketil | Hylland (Chair) | Norway; University of Oslo & IMR, Bergen |
| Natalja | Kolesova | Estonia; Taltech |
| Randel | Kreitsberg | Estonia; University of Tallinn |
| Ivan | Kuprijanov | Estonia; Taltech |
| Kari | Lehtonen (Chair) | Finland; SYKE |
| Ionan | Marigomez | Spain; UPV/EHU |
| Concepcion | Martinez-Gomez | Spain; IEO, CSIC |
| Aourell | Mauffret | France; IFREMER |
| Susana | Galante-Oliveira | Portugal; University of Aveiro |
| Halldor | Haldorsson | Iceland; University of Iceland |
| Joana | Raimundo | Portugal; IPMA |
| Milda | Stankeviciute | Lithuania; University of Vilnius & Nature Research Centre |
| Evita | Strode | Latvia; Latvian Institute of Aquatic Ecology |
| Joachim | Sturve | Sweden; University of Gothenburg |
| Zhanna | Tairova | Denmark; Aarhus University |
| Raisa | Turja | Finland; SYKE |