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► **To cite this version:**

Wilfried Sanchez, Jean-Marc Porcher. Fish biomarkers for environmental monitoring within the Water Framework Directive of the European Union. *Trends in Analytical Chemistry*, 2009, 28 (2), pp.150-158. 10.1016/j.trac.2008.10.012 . ineris-00961936

**HAL Id: ineris-00961936**

**<https://ineris.hal.science/ineris-00961936>**

Submitted on 20 Mar 2014

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**Fish biomarkers as a useful tool for environmental monitoring within the Water Framework Directive (WFD)**

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## **Abstract**

During the last 25 years, numerous biomarkers have been developed with the objective to apply them for environmental biomonitoring. Recently, the Water Framework Directive (WFD) specified monitoring programmes required to assess the achievement of good chemical and ecological status for all water bodies by 2015. This article reviews the potential of biomarkers for ecotoxicological status assessment in WFD monitoring programmes. For this purpose, we define the roles and the functions of biomarkers as biomonitoring tools. We highlight also the importance to define a clear reference system to be confident that biomarkers represent a quantitative assessment of contaminant effects.

**Keywords :** Water Framework Directive ; Environmental monitoring ; Ecotoxicology ; Biomarkers ; Fish

**Abbreviations :** AChE : acetylcholinesterase, ALAD : amino-levulinic acid deshydratase, BEST : biomonitoring of environmental status and trends, EROD : 7-ethoxyresorufin-O-deethylase, GSI : gonad somatic index, JAMP : joint assessment and monitoring programme, LSI : liver somatic index, MEDPOL : programme for the assessment and control of pollution in the Mediterranean region, MT : metallothionein, SPG : spiggin, VTG : vitellogenin, WFD : water framework directive.

## **1. Introduction**

The Water Framework Directive (WFD, 2000/60/EC) adopted by the European Parliament and the Council of the European Union in October 2000 will provide the major driver for achieving sustainable management of water in the Member States. The WFD establishes a framework for the protection of all water bodies, which prevents further deterioration of water resources, promotes sustainable water use and ensures the progressive reduction of pollution of water bodies. Overall, the WFD aims at achieving good chemical and ecological water status for all water bodies by 2015. For this purpose, article 8 and annex V of the WFD specify three monitoring regimes (Fig. 1) including,

- Surveillance monitoring, to provide information for the assessment of long-term changes in natural conditions, and changes resulting from widespread anthropogenic activity. Moreover, the results of this monitoring are used, in combination with the impact assessment, to determine requirements for future monitoring programmes.

- Operational monitoring, to establish the status of water bodies identified as being at risk of failing to meet their environmental objectives, and to assess any changes in the status of such bodies resulting from the programmes of measures.

- Investigative monitoring, to understand the causes of failure when operational monitoring showed that environmental objectives are not likely to be met, and to allow accidental pollution assessment.

These monitoring programmes must provide the information necessary to assess whether the WFD's environmental objectives will be achieved or not. For this purpose, several quality elements including chemical parameters and biological quality elements are clearly defined by the WFD.

Chemical status of the WFD is based on monitoring of priority substances identified as substances of concern at the European level according to the requirements of Art. 16 of the WFD (Table 1).

The achievement of good chemical status is based on compliance with environmental quality

standard defined at European level for each priority substance. This targeted approach provides valuable information on media contamination, but only for a limited number of chemicals.

Concurrently, ecological status is based on:

1/ the biological quality of all water bodies, which is mainly based upon composition, abundance, presence of sensitive taxa or diversity in various taxonomic assemblages including oligochaetes [2] and other benthic invertebrates [3], diatoms [4], and fish [5],

2/ hydro-morphological elements such as hydrological regime, river continuity and morphological conditions,

3/ chemical and physico-chemical elements including thermal conditions, salinity, acidification status and nutrient conditions, and also specific pollutants discharged into the water body.

Hence, surface waters can be classified into five classes of ecological status calibrated according to their deviation from reference conditions previously defined for a type of water body. The purpose of the ecological status assessment is thus to detect adverse ecological effects, integrating numerous stressors and acting at the community level.

Scientific researches in ecotoxicology have developed several methods such as *in vitro* bioassays, biomarkers, biosensors and whole organism bioassays, applicable in an environmental monitoring programme to complete the information provided by conventional environmental monitoring approaches [6] (Fig. 2). Among them, biomarkers are integrative tools that are believed to answer to WFD's challenges for improved detection of the impacts of chemical compounds on aquatic organisms, i.e. improved link between biological effects observed at the community level and monitored chemical concentrations.

Hence, it is envisaged that biomarkers will become in the future fully integrated in the monitoring programmes of the WFD, as part of the adaptation of the Directive to scientific and technical progress in accordance with the provisions of article 20 [1].

The aim of this review is to summarize the potential benefits from the implementation of biomarkers in the WFD. For this purpose, advantages and limits of biomarkers for environmental biomonitoring are described in the first section of this review. Enlightened by this information, the place that could be assigned to these emerging operational tools in the monitoring programmes embedded in the WFD is discussed. The last section presents the areas where further research is needed to increase the attractiveness of biomarkers for environmental monitoring and to bridge the gap between ecotoxicological research and policy demands for an effective implementation of the WFD.

## **2. Presentation of biomarkers for in situ trialing**

### **2.1. Definitions, utilities and limitations**

Biomarkers can be considered as complementary tools to chemical and ecological analysis classically used for field monitoring [7]. Firstly developed in human biology to provide an early diagnostic of pathologies, biomarkers were secondly used in ecotoxicology to assess the effects of pollution in wild organisms. In this context, a biomarker was defined as “a biochemical, cellular, physiological or behavioural variation that can be measured in tissue or body fluid samples or at the level of whole organisms that provides evidence of exposure to and/or effects of, one or more chemical pollution (and/or radiations)” [8]. To complete this definition, Van der Oost, [9] proposed several criteria to evaluate the strength and weakness of candidate biomarkers according to the work of Stegeman [10].

- The biomarker assays should be reliable, relatively cheap and easy to perform. Moreover, non-invasive or non-destructive methods should be selected preferentially to facilitate environmental biomonitoring in protected or endangered species [11].

- The biomarker response should be sensitive to xenobiotic exposure and/or effects to serve as an early warning parameter. Moreover, the temporal response profiles of biomarkers after exposure to chemicals should also be known for a better understanding of biomarker results [12].

- The impacts of confounding factors on baseline data and biomarker responses should be well established in order to distinguish between natural variability and pollution-induced stress. For this purpose, biology and physiology of selected organisms should be known to minimize variation sources (e.g. age, gender, reproductive status).

- The mechanisms supporting the relationships between biological responses used as biomarker and pollutant exposure should be defined, as well as the relationships between biomarker responses and impact to the organisms should be clarified.

Several biomarkers named core biomarkers are well described in scientific literature [13] and some of them may be used to assess the quality of aquatic environment (Table 1). However, due to the large number of pollutants encountered in aquatic environment and the various effects of these pollutants, no single biomarker can unequivocally determine environmental degradation. Hence, the application of a set of biomarkers based on complementary parameter measurements appears as a valuable way to differentiate clean and polluted sites or to describe accurately contamination effects on organisms [14-16].

## **2.2. Biomarker application in regulatory environmental monitoring networks**

Numerous scientific studies applied biomarker measurements in a biomonitoring context. These studies are geographically and temporally limited but provide valuable information to evaluate potential of biomarkers for environment quality assessment [9]. On the contrary, few data are available on the application of biomarkers in regulatory environmental monitoring networks (Table 2).

The Biomonitoring of Environmental Status and Trends (BEST) program of the US Geological Survey provides a nice application of biomarkers in large framework for freshwater monitoring [17]. This framework monitors water quality in large US river basins such as Rio Grande, Columbia or Yukon. For this purpose, several biomarkers including EROD activity and vitellogenin concentration but also lysozyme activity, macrophage aggregate analysis and histopathology were measured in multiple wild fish species. Fish health assessment and chemical monitoring in collected organisms complete biomarker analyses. This large national monitoring program provides geographic view of wild fish health and toxicological status but also it highlights the interest of biomarker measurements in freshwater biomonitoring context and shows that application of these ecotoxicological tools in national framework is possible. However, BEST data interpretation is complex due to effects of several confounding factors and argues for study design optimisation to minimize the effects of biotic and abiotic factors on biomarker responses (Joe E. Hinck, pers. comm.).

Environmental biomonitoring networks are also available to assess quality of marine ecosystems. Among these programmes, the Joint Assessment and Monitoring Programme (JAMP) was developed in the framework of Convention for the Protection of the Marine Environment of the Northeast Atlantic (the OSPAR convention). The aim of JAMP is to assess the concentrations, trends and effects of specific contaminants such as heavy metals, polyaromatic hydrocarbons and tributyltin, in the marine environment [18]. In this context, a set of biochemical parameters including EROD activity, cytochrome P450 quantification, AChE activity, vitellogenin, metallothionein, amino-levulinic acid deshydratase (ALAD) activity, lysosomal stability and DNA adducts were applied to monitor biological effects in various fish species such as dab, flounder, haddock or long rough dab [19]. These biochemical assays are completed by other end-points including liver and gonad somatic index (LSI and GSI respectively), condition factor, fish disease index, PAH metabolite quantification and histopathological analysis.

A set of biomarkers including EROD activity, metallothioneins, lysosomal membrane stability and DNA damages, was also used in the programme for the assessment and control of pollution in the Mediterranean region (MEDPOL) proposed in the United Nations Environment Programme. This biomonitoring network assists Mediterranean countries in the implementation of marine pollution trend monitoring, compliance monitoring and biological effects monitoring programmes. For this purpose, before monitoring activity, a quality assurance programme and an intercomparison exercise were set up to standardize methodologies employed in MEDPOL. Moreover, the proposed set of biomarkers can be implemented by participating countries to address specific end-points [20].

The JAMP and MEDPOL programmes provide large data set for the implementation of international biomonitoring networks based on biomarker assessment in aquatic organisms including fish and reflect national concerns for this application. However, the comparability of data appears as a major gap for the large application of a multi-biomarker approach and requires a comprehensive quality assurance programme [18].

### **3. Place of mechanism-based ecotoxicological tools in WFD monitoring programmes**

Peakall [21] proposed to substitute chemical analysis by measurement of specific biomarkers. However, this idea appears as in opposite with the increase of scientific knowledge. Several biomarker responses historically described as highly specific such as inhibition of acetylcholinesterase activity by organophosphorous pesticides or induction of metallothioneins by heavy metals, can be disturbed by other chemical compounds [22]. Moreover, relationship between biomarker response and chemical exposure is not strictly linear due to adaptive phenomenon [23] or transient response as reported for antioxidant parameters [24]. Difficulty to link chemical exposure and biochemical response is increased by pollutant interactions during exposure to binary or complex mixtures. Kirby [25] reported antagonist effects on EROD activity in flounder (*Platichthys flesus*) exposed to mixtures of polycyclic aromatic hydrocarbons and estrogenic

compounds. These results have been used to interpret EROD activity measured in wild flounder collected in United Kingdom estuaries and showed that a greater understanding of interaction that influence biomarker would be required to interpret monitoring data. In this context, application of a set of complementary biomarkers appears as relevant to highlight interactive effects as recommended by Sanchez et al. [26] and Aït-Aïssa et al. [27] that reported positive interactive effects respectively due to co-exposure to pesticides and alkylphenols, or estradiol and heavy metals mixture. While it is difficult to link chemical exposure and biomarker response, biomarker cannot be considered as an accurate chemical probe. However, biomarker application provides valuable and complementary information on bioavailability and metabolism of chemicals. Forbes et al. [28] argued that biomarkers did not provide relevant information on ecological effects able to appear after exposure to pollutants. Indeed, it is more difficult to link parameters reflecting distant biological organisation levels [29]. Any laboratory and field studies established correlations between distant parameters such as induction of cytochrome P450 and fish health index [29] or inhibition of vitellogenin and fecundity of fish [30]. However, it appears difficult to establish a clear relationship between biochemical responses and population disturbances. In a recent field study, Sanchez et al. [7] measured a set of biochemical biomarkers and physiological parameters in wild three-spined sticklebacks (*Gasterosteus aculeatus*), but also fish community endpoints. In Rhonelle river, fish were clearly affected by water pollution and fish assemblage was moderately disturbed and characterised by a clear decline of young stickleback number (Fig. 3). In this context, it could be possible to describe a causal link between biomarker responses and fish community disturbances. However, many environmental factors are able to disturb fish assemblage [31]. Only an integrated “weight-of-evidence” approach designed around the assessment of complementary parameters measured at various biological organisation levels could provide valuable data to improve a link between biochemistry and ecology [32]. This approach is not applicable in a large biomonitoring network due to practical and economical constraints. Hence, biomarkers cannot be

considered as a predictive signal but provide an early warning signal of fish health disturbance complementary to ecological monitoring.

Biomarkers appear as complementary tools to chemical and ecological approaches classically applied in aquatic environment monitoring. Hence, these parameters can be used in monitoring programmes required for the implementation of the WFD. In this context, biomarkers can allow to identify early biological effects [33] or contamination sources [15], to characterise mechanistic pathway between exposure and effects [34] and to help establish relationship between chemical and ecological status [9]. However, it is economically difficult to apply biomarkers extensively for all monitoring regimes required by the WFD. Hence, a rational application of these tools can be proposed.

Investigative monitoring programme aims at understanding the causes of such failure when environmental objectives are not likely to be met (Fig. 1). For this purpose, it will be specifically designed and focused on relevant quality elements. In this context, ecotoxicological monitoring based on biomarker measurement would be appropriate to integrate the effects of contamination on organisms and to drive further chemical analysis for a better environmental risk assessment.

Surveillance and operational monitoring programmes are designed to establish the status of all European water bodies based on physico-chemical, chemical and biological monitoring (Fig. 1). In these programmes, biomarkers could be used where the water body exhibited a good chemical status and a bad ecological status to indicate whether or not water quality constraints are restricting ecological status. Moreover, this approach can drive chemical analysis if a positive response is recorded for specific biomarkers such as endocrine disruption parameters. These specific parameters can be also proposed to apply a combined pressure and impact assessments able to support a better confidence in the “at risk” or “not at risk” designation of water bodies [35]. The authors described this potentiality around the example of tributyltin contamination and imposex in the gastropod snail *Nucella lapillus* but several applications could be proposed using other

biochemical biomarkers. For example, vitellogenin induction, a biomarker of estrogenic endocrine disruption [36], could be measured in combination with the chemical environmental quality standard defined for priority WFD substances with estrogenic activity such as nonylphenol, octylphenol or di(2-ethylhexyl)phthalate.

Practically, further applications of biomarkers in WFD monitoring programmes will require selecting species for biomarker measurement. Several species such as benthic invertebrates and fish are proposed for ecological assessment of freshwater ecosystems based on population monitoring. Hence, in a WFD context, it could be more interesting to combine ecological and ecotoxicological monitoring networks to decrease cost of directive implementation. In this context, fish appears as an interesting species to apply biomarkers. Indeed, many data on fish ecotoxicology are available in scientific literature and this species is more integrative of contamination due to its place at a higher level in the food-web. Hagger et al. [37] argued for biomarker measurements in a wide range of phyla exhibiting different feeding strategies to integrate different routes of exposure and interspecies variation in effects/susceptibility at a location. Moreover, this multi-species approach is also required to cover a large geographic scale due to European species distribution.

#### **4. What reference system can be used for ecotoxicological monitoring based on biomarkers ?**

Assessment of water status in the WFD is based on the extent of deviation from previously established reference conditions. They represent the best status achievable (i.e. the benchmark) and it is defined as the biological, chemical and morphological conditions associated with no or very low human pressure [1]. The reference conditions are type-specific, so they are different for different types of rivers, lakes or coastal water so as to take into account the broad diversity of ecological region in Europe. This concept appears also very interesting to assess ecotoxicological status of European water bodies using biomarkers. Indeed, it is crucial for the validity of the biological assessment to define reference conditions and to establish a classification that is effective

in detecting changes due to pollution by adequately separating natural variation from variation caused by anthropogenic impacts [38-39]. Several strategies are classically used to define a valuable reference system that allows biomarker data analysis.

- Definition of a relative reference allows to determine differential biomarker responses between an investigated site and an upstream site. This method can be used for upstream/downstream studies [40] or for stream profile characterisation [41]. However, it appears more difficult to apply this strategy on a large scale due to fish assemblage modifications in hydrographic network [42].

- Definition of a temporal reference is based on long term monitoring in the same site. This strategy allows to leave inter-site variability out of account but raises the question of old data validity due to inter-annual variation of biomarker levels [43].

- Definition of a reference in a low contaminated site without connectivity with other investigated sites is classically used to assess biomarker responses in a large scale [44-45]. However, this method requires a rigorous process to select reference sites due to site contamination and/or geographic variability as described by Mayon et al. [44].

In all cases, the defined reference is pragmatic. Hence, to be confident that environmental monitoring based on biomarkers represents a quantitative assessment, it appears necessary to know the range of natural variability of assessed parameters in healthy organisms [20,46]. This purpose requires to define natural biomarker variability due to biotic and abiotic parameters including respectively gender, age, reproductive status or genotype and temperature, turbidity, diet or sampling season (Fig. 4). However, this point can be considered as one of the most difficult aspect of in situ biomarker characterisation and few studies assessed this variability in wild model fish species [47]. This kind of work will allow the establishment of the normal physiological ranges of biomarkers associated to a safety factor as proposed by Schlenk [48] and Depledge [8]. These normal values could be used to develop specific tools for biomarker data analysis. Indeed, several

authors proposed index based on biomarker responses usable as a tool for environment managers to evaluate the relative environment hazard at investigated sites [35,49]. However, integration of normal physiological values in biomarker index integration process could allow development and validation of WFD compatible biomarker index based on deviation from reference concept.

## **5. Conclusion**

As presented in the present article, numerous biomarkers developed in ecotoxicology have been successfully applied in large monitoring network around the world (BEST, JAMP and MEDPOL programmes) to assess health and toxicological status of wild fish and could be used for the implementation of the WFD. Indeed, biomarkers are considered as an early warning signal that allows to evaluate the effects of contamination on the exposed biota and can provide valuable data to assess the ecotoxicological status of European water bodies. We believe these tools may bridge the gap between chemical monitoring and biomonitoring, more especially for investigative monitoring programme of WFD. Biomarkers may indeed help Member States to identify specific pollutants in cases when water bodies have a good chemical status but a bad ecological status. However, it is necessary to increase scientific knowledge for a better interpretation of biomarker data and also to be confident in biomonitoring data. This point requires more research into physiology, genetic, life-history traits of sentinel organisms and definition of natural variability range of biomarkers. Furthermore, biomarkers can be now applied in combination with chemical monitoring and biomonitoring but also with *in vitro* bioassays, gene expression tools or histological analysis in a “weight of evidence” approach able to improve environmental risk assessment in specific sites. Hence, in this context, further researches are needed to develop and to validate new biomarkers that integrate exposure to emerging contaminants and/or specific mechanisms of action of environmental pollutants.

## **Acknowledgements**

We thank V. Bonnomet, A. Morin and V. Dulio from INERIS for information on WFD. We thank also J.E. Hinck from U.S. Geological Survey for helpful information on BEST program. We acknowledge financial support of the French Ministry of Environment and Sustainable Development and the « Région Picardie ».

The views and opinions expressed in this publication are those of the authors alone and do not necessarily reflect those of the INERIS and the French Ministry of Environment and Sustainable Development.

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**Jean-Marc Porcher** (PhD) is a toxicologist and has 15 year research experience, focused at a developing biomarkers and in vitro methods to understand mechanisms of toxicity, detect toxicological effects at the laboratory and in the field and developing in vitro and in vivo models for identification of xenobiotics. He is currently head of Ecotoxicology research unit at National Institute of Industrial Environment and Risks (INERIS), France.

## Figure captions

Figure 1. Description of the different types of monitoring programmes for the Water Framework Directive (modified from Hagger et al. [37] and Allan et al. [6]). Surveillance monitoring programme concerns a selection of at risk and not at risk water bodies. Operational monitoring programme is focused exclusively on water bodies at risk. Investigative monitoring programme is focused on water bodies characterised by poor or bad status to identify sources of failures.

Figure 2. Position of biomarkers among other environmental monitoring methods according to their specificity, ecological relevance and temporal sensitivity (adapted from Adams et al. [50]).

Figure 3. “Weight of evidence” approach that combined chemical, biochemical, histological, population and community measurements, proposed by Sanchez et al. [7] to establish a link between chemical, biochemical, histological and population levels in freshwater sampling sites.

Figure 4. Presentation of biotic and abiotic parameters known to influence physiological range of biomarkers and influence their responses under chemical stress.

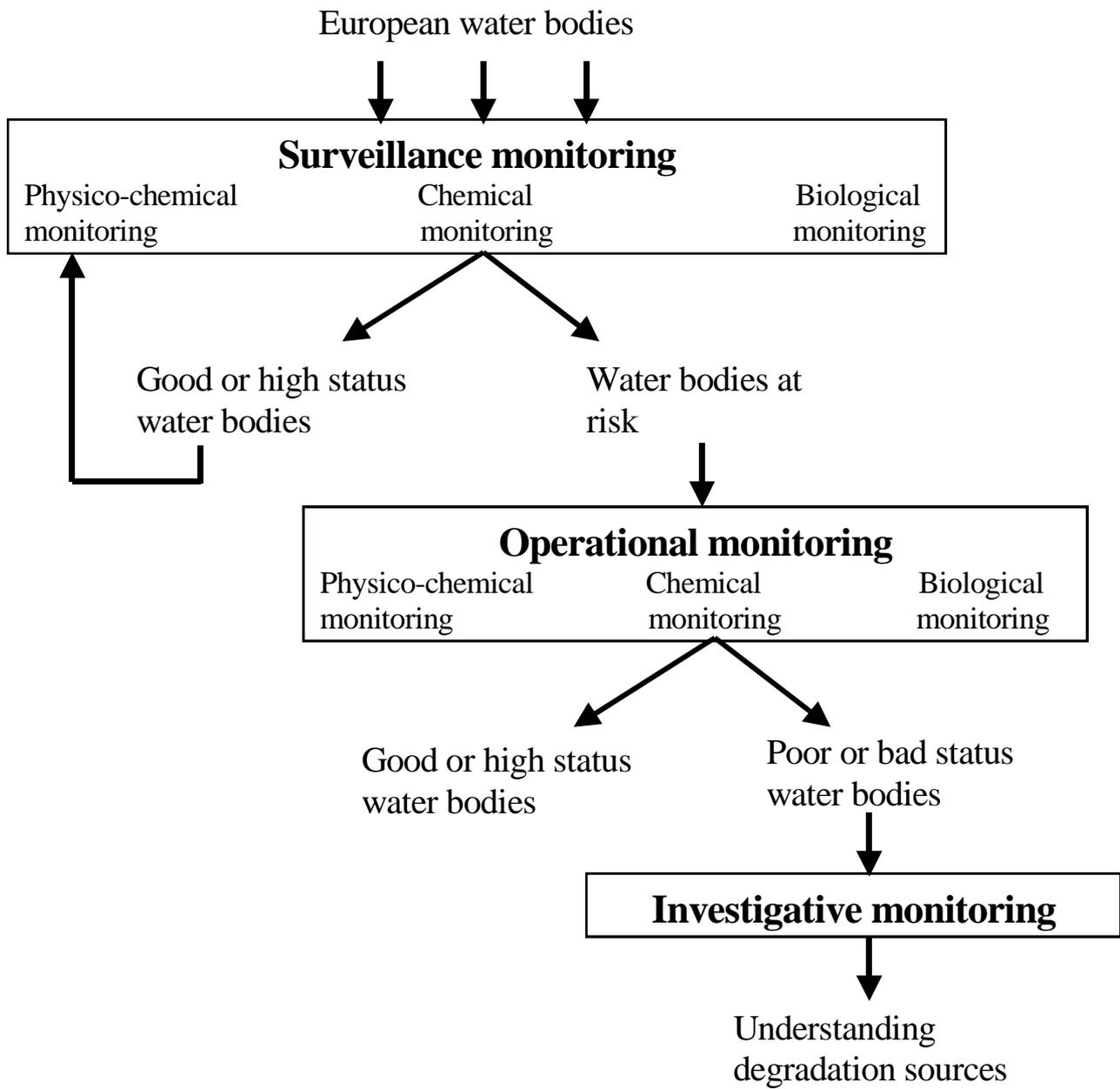


Figure 1.

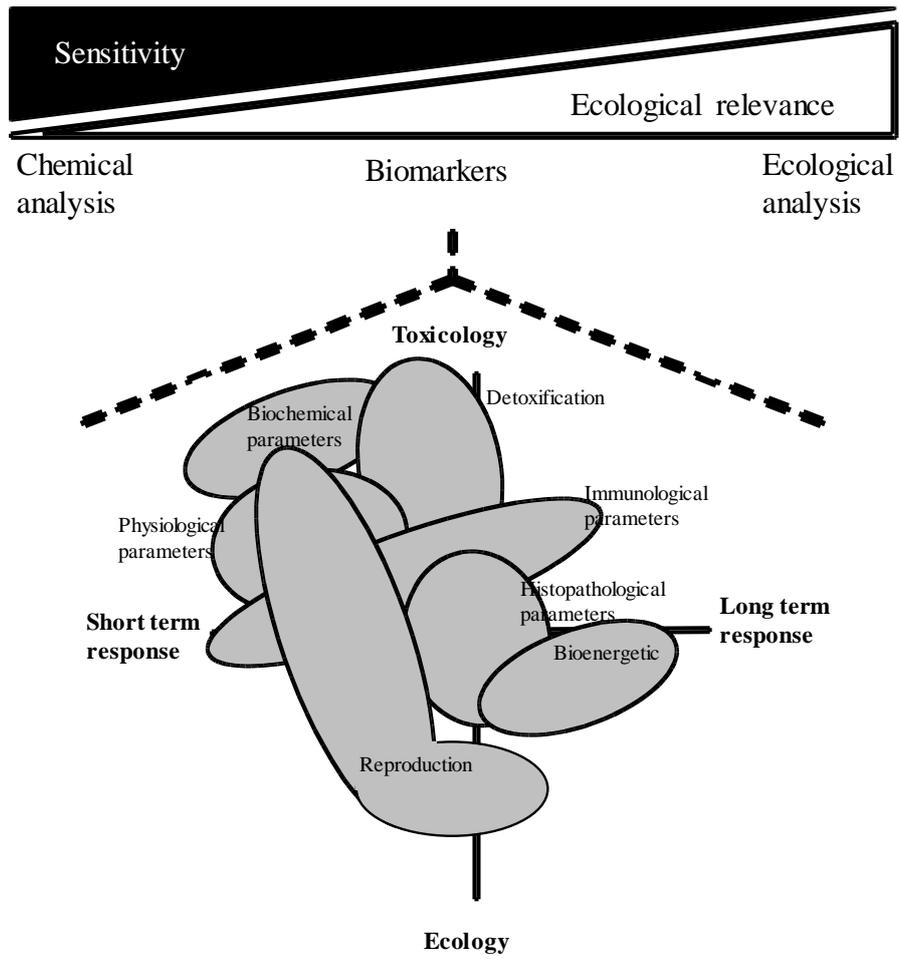


Figure 2.

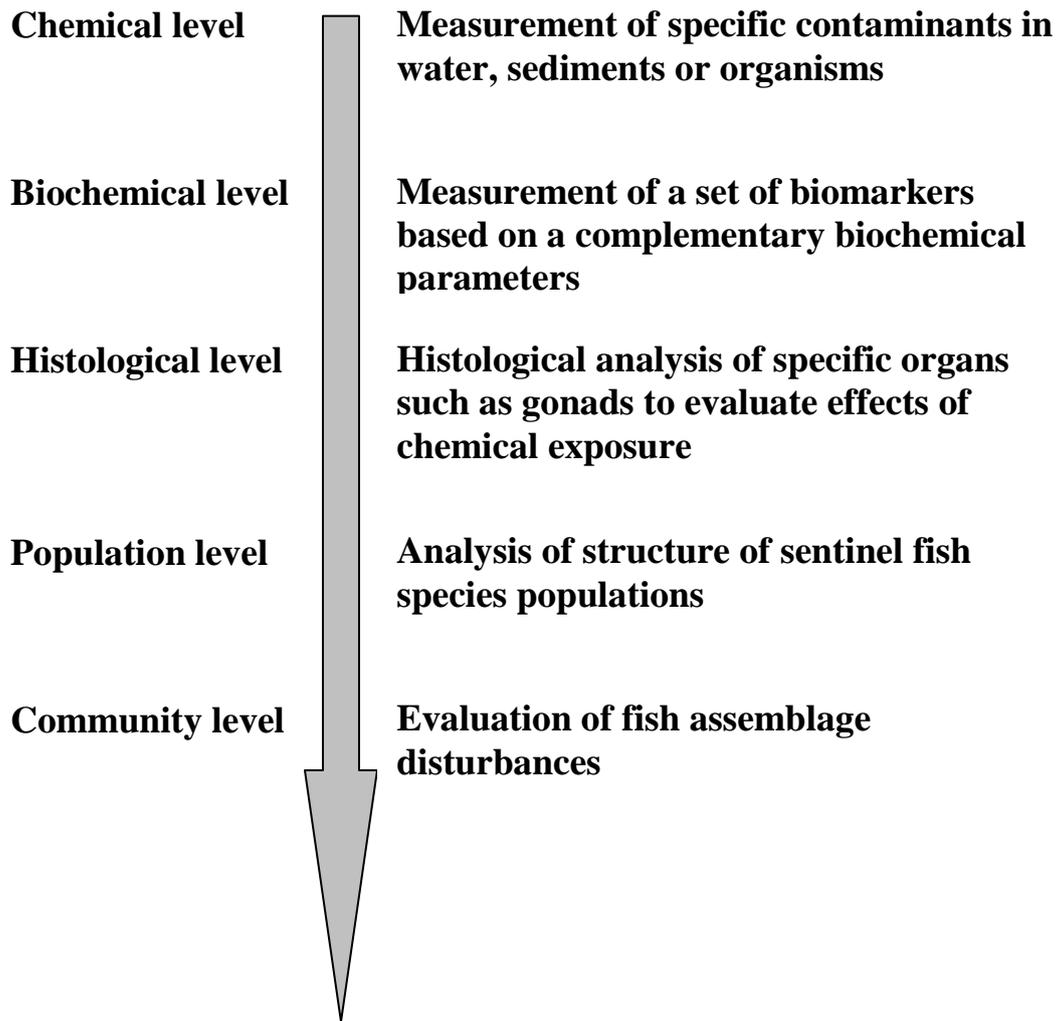


Figure 3.

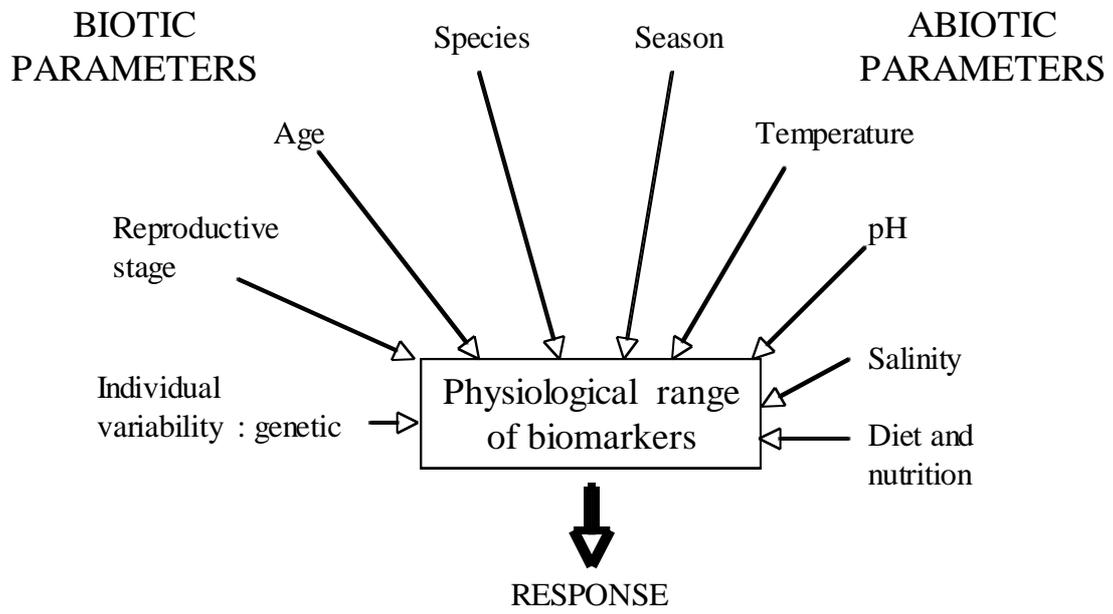


Figure 4.

Table 1. List of 41 dangerous substances used for chemical status definition including the 33 European priority substances and 8 other pollutants that derive from the "daughter directives" of the European Directive on Dangerous Substances 76/464/EEC.

Priority Substances			
1	Alachlor	22	Naphtalene
2	Anthracene	23	Nickel and its compounds
3	Atrazine	24	Nonylphenols
4	Benzene	25	Octylphenol
5	Brominated diphenylethers	26	Pentachlorobenzene
6	Cadmium and its compounds	27	Pentachlorophenol
7	C <sub>10-13</sub> -chloroalkanes	28 <sup>a</sup>	Polyaromatic hydrocarbons
8	Chlorfenvinphos	29	Simazine
9	Chlorpyrifos	30	Tributyltin and its compounds
10	1,2-dichloroethane	31	Trichlorobenzenes
11	Dichloromethane	32	Trichloromethane
12	Di(2-ethylhexyl)phthalate	33	Trifluralin
13	Diuron	34	Aldrin
14	Endosulfan	35	Dieldrin
15	Fluoranthene	36	DDT total
16	Hexachlorobenzene	37	Endrin
17	Hexachlorobutadiene	38	Isodrin
18	Hexachlorocyclohexane	39	Carbontetrachloride
19	Isoproturon	40	Tetrachloroethylene
20	Lead and its compounds	41	Trichloroethylene
21	Mercury and its compounds		

a. Polyaromatic hydrocarbons include benzo(a)pyrene, benzo(b)fluoranthene, benzo(g,h,i)perylene, benzo(k)fluoranthene and indeno(1,2,3-cd)pyrene.

Table 2. Presentation of core biomarkers used to assess health and toxicological status of wild fish in environmental monitoring networks.

<b>Biomarker</b>	<b>Monitoring programme<sup>a</sup></b>	<b>Description</b>	<b>Sensitivity</b>
EROD activity	J, M, B	Biotransformation enzyme induced by planar hydrocarbon	PCBs, PAHs and dioxin-like compounds
Acetylcholinesterase activity (AChE)	J	Enzyme implicated in nervous transmission	Organophosphates, carbamates and similar molecules
Vitellogenin (VTG)	J, B	A precursor of egg yolk, normally synthesized by female fish	Estrogenic endocrine disrupter compounds
Metallothionein (MT)	J, M	Metal scavenger implicated in protection against oxidative stress	Heavy metals and inducer of oxidative stress
Amino-levulinic acid deshydratase (ALAD)	J	Enzyme implicated in amino-acid metabolism	Lead exposure
Lysosomal stability	J	Lysosomes play a key role in liver injury caused by various xenobiotics	Overall organism health
DNA damages	J, M	Alteration of DNA structure able to disturb DNA function	Genotoxic compounds including PAHs and other synthetic organic
Lysozyme activity	B	Disease resistance factor	Overall organism health
Macrophage aggregate analysis	B	First line of immune defence for the organisms	Multiple contaminants including PAHs and metals

<sup>a</sup> B : Biomonitoring of Environmental Status and Trends (BEST), J : Joint Assessment and Monitoring Programme (JAMP), M : programme for the assessment and control of pollution in the Mediterranean region (MEDPOL).