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SCIENCE DIRECT
Talanta 69 (2006) 302–322
Talanta
www.elsevier.com/locate/talanta

Review

A "toolbox" for biological and chemical monitoring requirements for the European Union's Water Framework Directive

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Received 23 February 2005; received in revised form 26 July 2005; accepted 29 September 2005
Available online 15 November 2005

Abstract

Until now, water quality monitoring has relied heavily on spot sampling followed by instrumental analytical measurements to determine pollutant concentrations. Despite a number of advantages, this procedure has considerable limitations in terms of (i) temporal and spatial resolution that may be achieved at reasonable cost, and (ii) the information on bioavailability that may be obtained. Successful implementation of the Water Framework Directive (2000/60/EC) across EU member states will require the establishment and use of emerging and low-cost tools as part of monitoring programmes. These techniques may complement monitoring already in place by providing additional information with the aim to obtain a more representative picture of the quality of a water body.

This article considers the limitations associated with current monitoring practice and presents, in the form of a review, emerging biological and chemical monitoring tools that may become part of a 'toolbox' of techniques for use by those in charge of assessing water quality. Biological monitoring techniques include biomarkers, biosensors, biological early warning systems and whole-organism bioassays. Sampling and analytical tools developed for chemical assessment comprise biosensors, immunoassays, passive samplers, and sensors. Descriptions of these devices and a discussion of their suitability for different types of monitoring detailing advantages and limitations are presented. Finally, quality assurance and quality control or method validation issues are summarised.

Keywords: Water Framework Directive; WFD; Water quality; Chemical monitoring; Biological monitoring; Quality assurance; Sampling; Representativeness

Contents

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doi:10.1016/j.talanta.2005.09.043

1. Introduction

1.1. The Water Framework Directive

The European Union's Water Framework Directive (WFD) is one of the most important pieces of environmental legislation produced in recent years and is likely to transform the way that water quality monitoring is undertaken across all member states [1,2]. It aims to complement a number of other existing legislative instruments including the Bathing (76/160/EEC), Drinking (98/83/EC), Fish (78/659/EEC) and Shellfish (79/923/EEC) Water Directives, as well as those based on specific substances or sources of pollution (i.e. Dangerous Substances (76/464/EC), Groundwater (80/68/EEC), Nitrate (91/676/EEC) and Pesticide (91/414/EEC) Directives) [2]. The objectives of the WFD (2000/60/EC) [3] are to improve, protect and prevent further deterioration of water quality across Europe. The term "water" within the WFD encompasses most types of water body, and therefore the legislation applies not only to groundwater but also to all coastal and surface waters. The Directive aims to achieve and ensure "good quality" status of all water bodies throughout Europe by 2015, and this is to be achieved by implementing management plans at the river basin level. Monitoring is required to cover a number of 'water quality elements' including, physico-chemical, hydro-morphological, biological and chemical parameters. Chemical monitoring is expected to intensify and will follow a list of 33 priority chemicals (inorganic and organic pollutants and substances) that will be reviewed every 4 years. The environmental quality standards (EQSs) for these substances have yet to be stated [4].

Three modes of monitoring regime are specified in the Directive and will form part of the management plans that must be introduced by December 2006. These include:

(i) *surveillance* monitoring aimed at assessing long-term water quality changes and providing baseline data on river basins allowing the design and implementation of other types of monitoring,
(ii) *operational* monitoring aimed at providing additional and essential data on water bodies at risk or failing environmental objectives of the WFD,
(iii) *investigative* monitoring aimed at assessing causes of such failure.

Aquatic systems are complex and there are many problems associated with monitoring their quality. If good quality status is achieved only surveillance monitoring is required to ensure this is maintained. However, for water bodies which are determined to be at risk, or of moderate or poor quality, further information will be needed so that adequate remediation strategies can be implemented and subsequently monitored (Fig. 1). Each stage of the process shown in Fig. 1 requires the use of a suitable set of 'tools' to obtain meaningful and reliable data and indicates the extent and complexity of the information required for the successful management of water bodies. While most of the tools may be used for all types of monitoring (i.e. investigative, operational or surveillance), some may be more suited or specifically adapted to certain situations or sites. This choice will depend on their deployment characteristics, cost, robustness, sensitivity and the type of measurand and information required. The WFD does not mandate the use of a particular set of monitoring methods, but aims to ensure the establishment of an adequate monitoring programme based on the quality elements mentioned above. The additional cost of the monitoring necessary to underpin the Directive will be an important factor in determining the selection of particular tools. The successful implementation of the WFD will rely on the availability of low-cost tools and technologies able to deliver appropriate and reliable data. In addition, as many large river basins encompass a number of countries, it is important to ensure that the data collected by different EU member states are of comparable and appropriate quality [2,4,5]. To achieve this, new analytical methods, the production of relevant certified reference materials and the organisation of inter-laboratory trials and proficiency testing schemes will be required [4,6].

1.2. Aims and objectives of this review

This review is based on a technical report, a 'directory of emerging techniques and methods for water quality monitoring', recently completed under the European Union's Sixth Framework Project, "Screening Methods for Water Data Information in Support of the Implementation of the Water Framework Directive (SWIFT-WFD; www.swift-wfd.com) project". The directory aims to list the commercially available and prototype techniques or tools that may be considered for use in the water quality monitoring programmes necessary for the implementation of the WFD. This monitoring includes assessment of biological/ecological quality elements, chemical monitoring of both inorganic and organic priority pollutants and measurement of physico-chemical parameters.

The techniques currently available for the assessment of 'biological quality' include: biomarkers, whole-organism bioassays, and biological early warning systems (BEWS). Ecological monitoring is usually achieved using specific evaluation tools and indices. Methods currently employed for chemical monitoring generally rely on the collection of spot water samples and on-line or continuous monitoring. Emerging methods for this purpose include: biosensors, electrochemical sensors, immunoassays and passive samplers. A vast number of techniques is in use

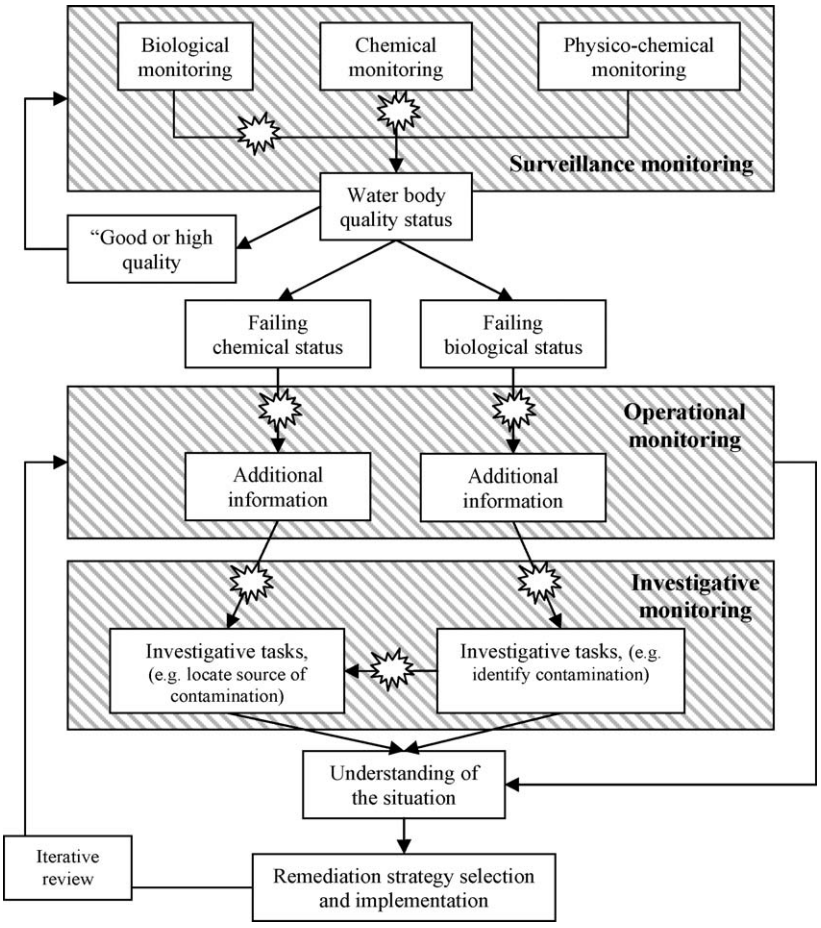

Fig. 1. Simplified scheme for the three types of monitoring embedded in the Water Framework Directive, namely surveillance, operational and investigative monitoring. The use of emerging tools and technologies is represented by the star (✵) symbols.

for the measurement of bulk physico-chemical parameters (i.e. conductivity, dissolved oxygen, nutrients and pH); although these are listed in the directory they will not be discussed further here.

The objective of this review is not to provide an exhaustive list of existing and emerging methods and techniques which could be used in the monitoring programmes. Rather it aims to assess the limitations of current monitoring practises and techniques and provide a balanced overview of the range of emerging tools available, focusing on their suitability for the types of monitoring embedded in the WFD, and to compare their relative advantages and disadvantages for this purpose. The last section of the review discusses the importance of precise analytical measurements, the development of quality control and quality assurance and validation schemes, and their application to the emerging technologies e.g. through the development of new reference materials and the establishment of European wide proficiency testing schemes.

2. Why using emerging tools for water monitoring?

According to the WFD, the deadline for all the monitoring programmes to be operational is December 2006 [2]. No one technology is suitable for this purpose. There is an urgent need to identify the most appropriate monitoring technologies from the wide range available for inclusion in the tool box to be used by those in charge of ensuring water quality across the member states.

Until now, monitoring of water quality has generally relied on the collection, at prescribed periods of time, of spot water samples followed by extraction and laboratory-based instrumental analysis for both inorganic and organic pollutants. In most cases the collected water sample is analysed directly to measure the 'total' concentration of a particular analyte. This methodology is well established and validated and therefore has been accepted for regulatory and law enforcement purposes. However, this approach is valid only if it provides a truly representative picture/status of the chemical quality of water at a particular sampling site. This is generally assumed. Research during the last two decades has shown that considerable limitations are associated with spot sampling to determine total pollutant concentrations [7]. Fig. 2 indicates where standard spot sampling/chemical analysis stands in relation to an inter-related scheme of emerging tools that could be used to monitor the source, pathway and sinks of environmental contamination.

An important number of factors is not accounted for by spot sampling. Metal speciation is one of these and has been shown to

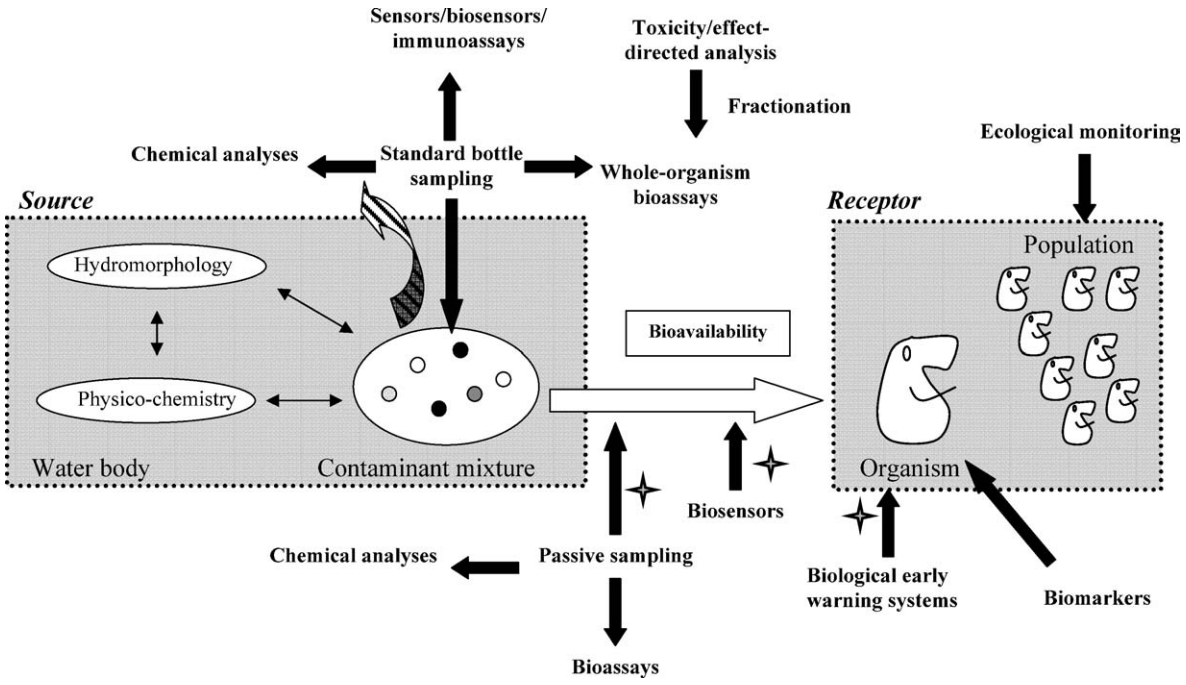

Fig. 2. Suitability of existing and emerging techniques and methods for water quality monitoring under Water Framework Directive. Thin arrows represent the interaction of the hydromorphology, physico-chemical properties of a water body with contaminants present in the water. Thick arrows represent possible monitoring strategies that may be employed to assess ecosystem health and water quality, while the four-point stars (✦) and the curved arrow (↷) represent sampling methods that may incorporate an additional temporal dimension and standard spot sampling, respectively.

be a crucial factor in metal toxicity to aquatic organisms [8–10]. For many metals it is now recognised, that according to the free-ion activity model, it is the free-ion fraction that is responsible for the observed toxicity [11]. Metal competition with naturally occurring cations, complexation with organic ligands, association to dissolved organic carbon (DOC) or colloids, or sorption to suspended sediment particles are processes that may be contributing to a reduction in metal bioavailability and toxicity in aquatic systems [9]. Such factors need to be accounted for during both sampling and subsequent sample extraction steps. Similarly, for hydrophobic organic pollutants, sorption to DOC or colloidal matter and sediments may significantly alter their bioavailability [12]. Therefore, sample acidification for metals and extraction of whole-water (i.e. both suspended solids and water) samples for organic chemicals aiming to obtain 'total' concentrations do not necessarily provide a representative picture of the level of pollution [12]. In these cases, if whole-water total concentrations form the basis of EQSs, then monitoring programmes should also focus on measuring pollutants in bed-sediments and biota [13]. It is expected that for priority metals, EQSs and monitoring will focus on the dissolved fraction, while for organic pollutants, the whole water (dissolved + sediment-bound fractions) should be considered. For hydrophobic compounds, suspended-, bed-sediment and biota may need to be monitored [4,14].

A further factor is that continuously varying hydromorphological, and hydrological conditions and intermittent chemical releases associated with industrial/urban wastewater effluents, bed-sediment re-suspension and diffuse pollution (e.g. run-off from the periodic application of pesticides to agricultural land) lead to spacio-temporal variations in a water body's physico-chemical characteristics [12,15]. For example, the temporal variations in the concentration of the herbicide diuron in the Maas River (continuously monitored at Eijsden field station in The Netherlands) over the period 2000–2005 (www.aqualarm.nl) showed that concentrations can vary by orders of magnitudes with time (Fig. 3). The peak levels follow the seasonal pattern of application of this herbicide.

Spot water sampling therefore provides only a 'snapshot' of the situation at the set time of sampling and fails to provide information on the bioavailability of pollutants in water. A summary of the limitations of spot sampling is given in Table 1. A 'toolbox' consisting of a range of existing/emerging techniques and methodologies may give additional information in order to obtain a clearer picture of the biological and chemical quality of a water body (Table 2). Fig. 2 outlines how these different approaches to monitor the water quality complement one another, and when used together, provide a more representative picture of the system under study.

It is clear the WFD will rely on the effective use of a combination of monitoring methods according to their suitability for the questions being asked and characteristics of the given site of sampling. The use of repeated spot sampling alone would be very expensive because of transport and analytical costs. Deployment of time-integrated sampling systems e.g. passive samplers [16] based on the uptake of truly dissolved contaminants or the establishment of continuous monitoring stations with both biological and chemical testing capabilities [17,18], may provide, at lower

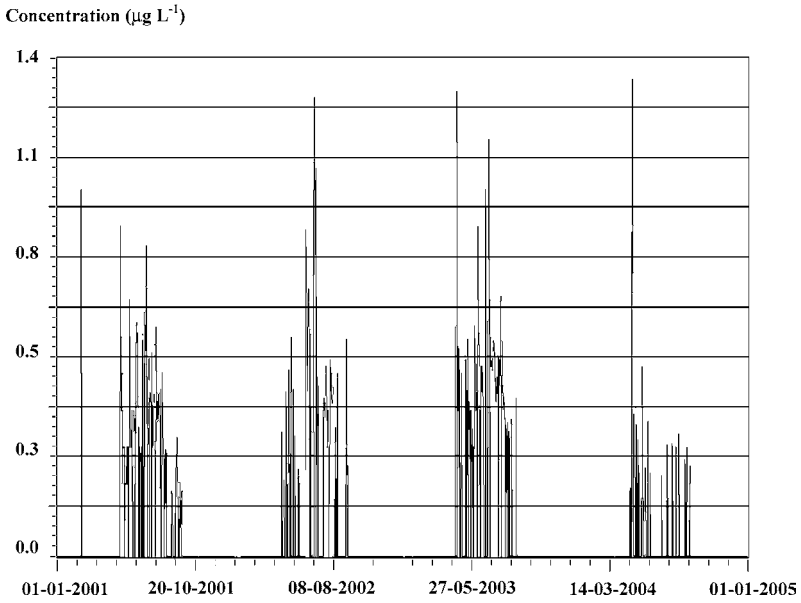

Fig. 3. Temporal variations in diuron concentration ($\mu g\,L^{-1}$) continuously monitored in the Maas River water at RIZA's Eijsden monitoring station for the period 2001–2005.

cost, more useful data on the variability of contaminant concentrations or temporal changes in toxicity. Ecological monitoring [19], biomarkers [20] or bioassays [21] may be useful in providing a more realistic assessment of impacts and exposure of aquatic organisms to specific contaminants or a mixture of contaminants present in the water. However, water quality managers and legislators need to be aware that these different methods measure levels of a pollutant within different fractions of the overall sample of water (Fig. 2 and Table 2). Dissimilar answers for variants of passive samplers, spot samples or filtered spot samples, in situ techniques, on-line or laboratory-based tools are to be anticipated [22]. This will also have implications in terms of regulatory analysis and method validation. Biological and chemical monitoring alternatives to spot sampling are reviewed in the following sections.

3. Biological monitoring

The use of whole-organism assays and the measurement of various biological responses provide an approach for the assessment of the quality of a water body. This approach has taken on renewed importance as the aquatic fauna are the primary recipients of water pollutants. Biological monitoring may be performed at a number of levels. At the cellular and intracellular levels specific biomarkers, sensitive to the early detection of degradation of water quality, can be measured [23]. Whole-

Table 1
Rationale for the updating of water quality monitoring and corresponding technologies aiming to rectify these insufficiencies

	Rationale	Appropriate emerging technologies
1	Standard spot sampling is costly and labour-intensive	Passive samplers, immunoassays, sensors/biosensors
2	Chemical monitoring based on spot sampling fails to detect and account for temporal variation in pollutant concentrations: It fails to provide a truly representative picture of the extent of contamination	Passive samplers, continuous monitoring equipment (e.g. SAMOS), certain on-line sensors/biosensors, biological early warning systems (BEWS)
3	The collection of bottle or spot samples allows the determination of total contaminant concentrations: fails to account for the bioavailability of pollutants in water (especially for non-polar organics and certain heavy metals)	Biosensors, passive samplers, and in certain circumstances immunoassays
4	Certain situations/sites such as drinking water intakes or wastewater effluents require results from monitoring to be obtained rapidly, however, standard spot sample collection, transport to the laboratory before processing and analysis is a lengthy procedure	BEWS, on-line monitoring systems, sensors or biosensors
5	Standard chemical monitoring can deliver important information on chemical levels for many pollutants, but it fails to provide any information on the toxicity of water samples	BEWS, biosensors, biomarkers and whole-organism bioassays
6	Screening methodologies including sampling and analytical steps need to be implemented by relatively unskilled monitoring personnel	Immunoassay test kits, passive sampling, bottle sampling, whole-organism bioassays, certain sensors and biosensors
7	At present, water quality monitoring does not rely on ecological and biological monitoring, however, a greater role is needed to assess ecological and biological integrity of water bodies, and use biological information as an early warning for system disturbances	Biomarkers, ecological monitoring and their combination

Table 2
Characteristics of the main types of prototype or commercially available tools and technologies for chemical and biological monitoring requirements within the WFD

Tools	Principle	Value measured	Deployment characteristics	Applicability	Advantages	Drawbacks
Water quality evaluation software	Assessing water quality based on physico-chemical measurements and benthic fauna assemblages and composition	Deviation from expected pristine condition for specific conditions of a particular site	Spot sampling followed by laboratory analysis	Freshwaters, rivers and lakes/estuaries/sea waters		
Biomarkers	Any biological response to an environmental chemical(s) at the sub-individual level, measured within an organism and its products	Chemical or pollutant concentrations	Spot sampling followed by laboratory analysis	Most types of waters	Early detection of contaminant impact and interaction with receptor organism	Need to account for the influence of their biological function
	Indicators of toxicity, exposure and susceptibility	Physiological and biochemical alterations specific to classes of pollutants		Many pollutants		Sometimes, need comparison to reference site
Whole-organism bioassays	Test based on the reaction of whole-organisms to toxicants present in water samples	Acute toxicity (including. geno-toxicity, cyto-toxicity or mutagenicity)	Laboratory and spot sampling based assays (a few in situ methods)	Most types of waters including groundwater	Very useful as preliminary screening devices	Only provide information on the acute toxicity of samples
					May be combined with toxicity directed analysis schemes	Results after 24–72 h
Biological early warning systems	Whole-organism bioassay specifically adapted to real-time measurements based on behavioural changes	Acute toxicity	On-line, in situ at secured sites	Most types of waters	Use of different trophic levels	Need energy supply
				Monitoring at remediation sites		Fails to provide longer term toxicity information
Spot sampling + chemical analysis	Collection of a water sample followed by extraction/filtration and chemical analysis (GC, ICP–MS)	Total contaminant concentrations	Bottle sampling	All types of waters	Easy to defend in court	Labour-intensive
				Most chemicals	Accuracy may be determined relatively easily	Provide a snapshot of the situation at sampling time
						Does not account for bioavailability

Table 2 (*Continued*).

Tools	Principle	Value measured	Deployment characteristics	Applicability	Advantages	Drawbacks
Continuous monitoring	Chemical analysis of continuous on-line water or 24 h composite samples	Pollutant concentrations	On-line at secured sites	Many organic pollutants	Rapid warning of concentrations exceeding EQSs	Need power supply and laboratory set-up at secured site
Passive samplers	Bio-mimetic sampling to mimic bioaccumulation or based on contaminant diffusion-limited accumulation into samplers	Bioaccumulation in aquatic organisms or truly dissolved time-averaged pollutant concentrations	In situ deployment at secured/unsecured sites and laboratory analysis	Most types of waters Priority pollutants (inc. polar/non-polar organics metals and heavy metals	Needs no energy supply	Bio-fouling problems
				Most types of waters	Deployment times from days to months Suitable for most types of waters - Inexpensive	Need for extensive laboratory calibration
Biosensors	Analytical device incorporating a combination of a specific biological element (creating a recognition event) and a physical element (transducing this event)	Total and bio-available pollutant concentrations	In situ, laboratory-based and continuous monitoring	Priority pollutants	May be based on continuous and on-site monitoring	Often requires skilled operators
		General toxicity, geno-toxicity and cyto-toxicity measures (BOD)		Organic and inorganic pollutants		Not applicable to all pollutants
Sensors	Detection and quantitation based on physico-chemical characteristics of contaminants	Contaminant concentrations	In situ, laboratory-based and continuous monitoring	Most types of waters Heavy metals, PAHs, and certain pesticides	Handheld instruments	Not applicable to all pollutants
Immunoassay test kits	Highly selective pollutant extraction and/or quantitation based on antigen/antibody interactions	Pollutant concentrations	Field or laboratory assays based on spot-sampling	Many organic pollutants e.g. pesticides, PAHs	Rapid and easy to employ Very sensitive, selective, rapid and inexpensive assays	Unit: analyte equivalents
				Certain metals	Easy to employ Ability to process many samples	Cross-reactivity with analogues and metabolites False positives Positive results may require further analysis

organisms can also be used in standardised toxicity tests, or by their integration into devices specifically designed to detect physiological and behavioural changes when the test species are subjected to a pollution event. At the highest level, the measurement of flora and fauna populations and communities forms an integral part of ecological status monitoring [19,24,25]. This is usually achieved by the use of commercially available evaluative software packages (e.g. RIVPACS), however, this is outside the scope of this review.

3.1. Biomarkers

A biomarker is defined as a change in a biological response (ranging from molecular through cellular and physiological responses to behavioural changes) which can be related to exposure to or toxic effects of environmental chemicals [26]. According to the World Health Organisation, biomarkers can be sub-divided into three classes (Table 3).

Biomarkers of exposure cover the detection and measurement of an exogenous substance, its metabolites, or the product of an interaction between a xenobiotic agent and target molecules or cells, in a compartment within an organism [27,28]. Molecular biomarkers of exposure are mainly composed of proteins, the functions of which ensure cell protection against potential toxic damage. This category includes membrane transporters involved in the eviction of toxic molecules outside the cell, proteins capable of metabolising xenobiotics, and chaperon-proteins involved in the detention of toxic molecules.

Numerous studies have shown interest in using heat shock proteins (HSP) for the detection of environmental stress at the cellular level. The expression of stress proteins, as HSP, is activated by thermal shock but also by a large variety of environmental conditions such as hypoxia/anoxia [29], osmotic pressure [30], presence of oxidizing agents, heavy metals and other toxic compounds [31–33]. The cytochrome P450 (CYP) family of proteins also represents another suite of potential environmental biomarkers. Inducibility of the expression or the activity of CYP is used to indicate contact or contamination with toxins, particularly polycyclic aromatic hydrocarbons, polychlorinated biphenyls, dioxins and pesticides [34–39]. Exposure to metals and heavy metal pollution is easily detected by the induction of metallothionein (MT) synthesis. MTs are involved in essential heavy metal homeostasis [40], cellular protective mechanisms after toxic metal exposure [41–43] and have a redox activity and antioxidant function [44,45]. MT inducibility under toxic conditions has allowed their use as toxicity biomarkers in numerous environmental studies [46–48].

Table 3
Examples of biomarkers and their applications [49]

Biomarkers	Pollutants
Biomarkers of exposure	
HSP	Thermal shock, metals/heavy metals,
Cytochrome P450	PAHs, PCBs, dioxin
Metallothionein	Metals
Glutathione S transferase	Hydrocarbons, PCBs, organochlorines
Biomarkers of effect	
Lysosomes	Stress
Antioxidant enzyme	PAHs, PCBs, organochlorine pesticides organophosphorus compounds, carbamates
Acetylcholinesterase	Endocrine disruptors
Vitellogenin	Endocrine disruptors
Biomarkers of susceptibility	
Paraoxonase	Organophosphates
Aryl human Receptor	PAHs

Biomarkers of effect include measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognised or associated with an established or possible health impairment of health or disease. Molecular biomarkers of effect indicate an infringement of the integrity of cellular physiology under the influence of drugs or xenobiotics. Integrity of cellular membranes (peroxidation of lipids), intracellular redox state, or the integrity of DNA molecules can constitute biomarkers of toxic effects. These biomarkers are very often correlated to concentrations or exposure time to a cytotoxic pollutant.

Lysosomes are used as biomarkers of environmental effects (stress) because they are involved in the uptake and accumulation of xenobiotics which in turn provoke measurable changes in the volume, size and number of lysosomes present. These biomarkers may be compound-specific (e.g. to PAH, PCBs or metals) or non-specific (generalised pollution of a water body) [20,49–51]. The decrease in the activity of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase or glutathione reductase can also be used to study the effect of PAHs, PCBs, and organochlorine pesticides on aquatic organisms [52–54].

Acetylcholinesterase (AChE) activity is inhibited in the presence of organophosphorus compounds and carbamates, and may be used to detect these molecules in seawater [55–57]. Induction of vitellogenin in male species of test organisms is also a good biomarker of effect of the presence of hormonal (endocrine disrupting) compounds in water [28,58–65].

Biomarkers of susceptibility indicate the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific xenobiotic substance, and include genetic factors and changes in receptors that alter the susceptibility of an organism to the exposure. This type of biomarker has been mostly investigated in the medical field. However, paraoxonase gene (PON1) (a liver and plasma enzyme involved in lipids oxidation) was identified as one of the first environmentally relevant genes when PON1 expression was discovered to be an important and sensitive marker for sensing exposure to organophosphates (OPs) [66]. Several research programmes have tried to confirm that aryl human receptor (AhR) expression levels correlate with PAH bioactivity.

The use of molecular biomarkers as a measure of toxicity requires an understanding of signal transduction or protection mechanisms involved in cells after contact with a substance or mixture of substances. Consequently, exposure can be associated with induction and variations in gene expression or with the modulation of enzymatic activity. The main interest in the use of molecular biomarkers resides in their ability to act as early alert signals, since toxicants have an impact at molecular and subcellular levels before their effects are observed at the

whole-organism and population level (Fig. 2). Moreover they are sensitive to concentrations below those causing cyto-toxicity.

In recent years, genomics has been applied in the areas of toxicology and ecotoxicology. The new field of toxicogenomics encompasses the study of the application of genomic tools to the detection of exposure to toxicants and ecotoxicogenomics is concerned with the responses at the genetic and protein level in organisms collected from the field after exposure to pollutants [67]. The main advantages lie in the possibility of testing many responses simultaneously because several thousand genes can be treated and subsequently monitored with the aid of high throughput sampling technology. Today, DNA chips and DNA microarrays allow the screening of many samples with a characterised marker [68,69] or can be used to aid in the identification of new ones [70].

Within the context of the WFD, it is envisaged that biomarkers will become important tools for investigative and operational monitoring. The study of biomarkers aims to give a quick response to a risk of pollution allowing rapid decision making. Their use, however, needs to be accompanied by an understanding of the significance of these measurements to ensure the adequate and reliable interpretation of these results by water quality managers. Once this initial step is achieved it may be possible to include biomarkers in monitoring for regulatory purposes [71]. An understanding of the characteristics of the sampling site, appropriate quality controls and replication, and seasonal and temporal variability of the test species deployed are important factors for correct interpretation of measurements [71]. Low cost and easy to use biomarkers need to be developed and tested [72]. It is important to ensure that such biomarkers operate at a range of trophic levels and are appropriate for different chemical substrates and types of water to ensure their widespread adoption. To date, biomarkers have shown potential as sensitive tools for the detection of pollution and it expected they will have their place amongst the tools water quality managers will utilise in the future [57,72,73].

3.2. Whole-organism bioassays

A whole-organism bioassay relies on the measurement (as acute or chronic toxicity) of the biological response of a test organism to a mixture of contaminants present in a water (e.g. drinking, ground, surface or wastewater effluent) sample in a standardised test usually conducted in the laboratory [21]. The observed toxic impact is generally the result of the bioavailability of the complex mixture of pollutants that may be present in the sample but is also dependent on physico-chemical parameters (e.g. DOC content, pH) of the water. A number of test species covering most of the different trophic levels in freshwater and/or estuarine/marine environments may be employed [74]. The use of multiple test species and trophic levels may be crucial for obtaining meaningful results or for fingerprinting, since many inter-comparisons of biological assays have shown differences in sensitivity to different chemicals or classes of compounds [21,75]. When conducting tests using microorganisms found at the base of the food chain, (e.g. *Vibrio fisheri* [Microtox® from Azur Environment], *Pseudomonas putida*, or microorganisms present in activated sludge), the test parameters usually measured are bioluminescence, metabolic status or growth, respectively [74]. *Vibrio fischeri* bioluminescence inhibition is the most common test, is relatively simple to implement, and a large database of results for many chemicals has been constituted. As standard ISO 11348 protocols exist for this assay, many commercial devices are available. A number of phototrophic organisms such as green algae, *Selenastrum capricornutum*, or *Pseudokirchneriella subcapitata* may be used following standard protocols [76], or using down-scaled micro tests [77] allowing the processing of a larger number of samples. Parameters frequently measured include the reduction in photosynthetic activity (by measuring fluorescence) or growth rate inhibition. More specific investigations into chlorophyll fluorescence ratios may allow detection of specific effects of herbicides which can affect either photosynthesis systems I or II [78]. Tests are routinely performed in glass flasks or by micro-plate assays for a period of 48–72 h [79,80]. Although the costs of the cell culturing facilities to set up the tests are relatively high, these may be reduced by the use of micro-biotests or tox-kits [81]. The use of dormant organism technology (e.g. algae or daphnid (*Daphnia magna*)) allows a simplified, rapid and cost-effective test without the inconvenience of cell cultures [82,83].

Chronic toxicity testing using invertebrates is common. Here the tests usually assess growth rate or survival of amphipods (e.g. *Hyalella azteca* or *Gammarus*), chironomid larvae (*Chironomas riparius*), daphnids, oysters (*Crassostrea gigas*) and many other organisms under controlled conditions [74,84]. Higher organisms such as fish are routinely used for risk assessment purposes in 96 h exposure trials. Toxicity endpoints used in these assays include larval/embryonic development rate, fish lethality or growth rate [21]. Other endpoints involving biochemical analysis are discussed in the section on biomarkers.

In order to enable the implementation (partly to replace standard expensive chemical analysis [85] and adoption by all member states of whole-organism bioassays in regulatory monitoring, the tests need to be simple to undertake, follow standardised protocols, be economical and predictive, and applicable to species, population and communities. In addition, they need to exhibit a wide range of sensitivities to multiple chemicals with minimal matrix effects [86,87].

Within the WFD monitoring programmes, bioassays may be used with the aim of controlling the toxicity of wastewater treatment effluents, changes in toxicity after accidental spills or to determine the source of a pollutant [88,89]. Many of these assays are available for application to sea and surface water samples, wastewater in/effluents and more generally to any water body ‘at risk’ [74,90]. While in certain circumstances (e.g. waste waters or accidental spills), toxicity may be sufficiently high to observe significant effects, many surface water samples may need preconcentration (e.g. on a chromatographic column of XAD resin), before significant toxicity can be detected [85,91]. However, in these cases there is a need to ensure that sample integrity is preserved if meaningful data is to be obtained.

Alternatively, and particularly applicable to investigative monitoring, toxicity/effect directed analysis (EDA) maybe undertaken to identify (using toxicity identification evaluation

(TIE) schemes), causes of observed toxicity in standard bioassays [92–95]. The extraction of samples using specific solid-phase extraction (SPE) columns [91], the addition of EDTA, or the alteration of pH, are some of the possible manoeuvres to remove and concentrate the organic (hydrophobic or hydrophilic) fraction, remove the metal fraction or investigate the effects of pH, respectively, on toxicity [94]. EDA can make use of chemical analysis to characterise the toxic components of complex mixtures [85]. In situ TIE including on-line exposure chambers for *Daphnia magna* coupled to sorbents for ammonia (zeolites), metals (Chelex) or organic chemicals (Ambersorb) have been the focus of recent research and have shown improved sensitivity compared to the US Environmental Protection Agency standard laboratory-based TIE schemes [92,93].

Importantly, most of these tests not only account for the presence of pollutants in the water sample but also for their bioavailability/bioaccesibility and physical transfer into the test organism [96]. However, as these assays are based on the collection of spot samples (which in itself represents a high initial cost), sample collection, preservation and assay time will affect sample integrity, e.g. by sorption of analytes to container walls or non-constant test concentrations [97], selective retention of organic compounds depending on their hydrophobicity and the type of SPE column used [91]. As part of an integrative risk assessment, in situ bioassays, such as an algal test based on the inclusion of *P. subcapitata* into alginate beads immobilised in a specifically designed apparatus, may provide an alternative to laboratory-based testing [98]. Another alternative biological monitoring approach is the use of in situ or continuous (on-line) biological early warning systems, overcoming problems associated with the collection of spot samples.

3.3. Biological early warning systems

Biomonitoring using biological early warning systems (BEWS) is based on the toxicological response of an organism to a contaminant or mixture of contaminants [17,99]. An acute toxicity measurement based on physiological or behavioural changes is used to provide a rapid warning in response to a deterioration in water quality [99]. A number of organisms have tentatively been used as BEWS and include fish species [100–102], daphnia, midge larvae, microorganisms (e.g. algae and bacteria) [103,104], or bivalve molluscs (e.g. various species of mussels) [105]. In some situations a combination of these test organisms has been used [106,107]. These on-line continuous (real-time) systems provide a rapid evaluation and detection of temporal variation in water quality and toxicity that cannot be achieved through standard approaches to chemical monitoring. Applications of BEWS include monitoring of drinking water intakes, water distribution systems, wastewater effluents, effluents from contamination remediation sites (where a rapid sensing of a change in water quality is needed) [108–111], or in river basin monitoring programmes [112]. These BEWS differ from biosensors by conserving the integrity of the whole-organism, rather than, for example, being based on a specific biological event within a cell of an organism. BEWS are generally constituted of a living organism, a sensing element to detect changes in the test organism, and a processing element to translate the signal from the sensing element into a warning response system. In many cases, monitoring is based on the use of a multitude of individuals of the same species/age group [105]. Sensitivity of a BEWS can be enhanced by increasing the resolution in the detection of a sufficiently significant magnitude of change in physiology or behaviour, or by measuring apparent but non-significant changes in a sufficiently high number of individuals. The secondary sensing systems used can be electric, electro-magnetic or optical signals, based on video-recording or chemical detection [99]. Fish monitoring systems usually make use of avoidance behaviour where fish species are positioned in a dual-fluvarium set-up comprising an uncontaminated stream and the water stream to be tested. Swimming and positioning behaviour or ability to swim against current may also be used in on-line biological monitors [99,113]. Ventilation monitors are based on the gill movement response to toxicants, and the measurement of ventilation frequency [100,101] is usually the most reliable and sensitive. The amplitude or the rate of 'coughs' may also be measured [114,115]. The species commonly used are rainbow trout (*Oncorhynchus mykiss*) or bluegill (*Lepomis macrohirus*) [115]. The secondary sensing system is composed of electrodes immersed close to the fish to monitor changes in electrical voltage associated with gill muscle activity [99].

Algal monitors (e.g. DF-Algentest), generally rely on fluorescence or oxygen production measurements to detect effects from herbicide or other toxicants interacting with chlorophyll photosynthetic systems [99]. However, the effect of contaminants on algal cell integrity may also be assessed through growth rate monitoring such as in the cage culture turbidostat [116]. BEWS based on the use of microorganisms usually involve the measurement of growth rates [104] or their ability to consume a metabolite e.g. on-line biological oxygen demand (BOD) sensors and allow the use of species that may be able to survive in saline and freshwater [103]. Invertebrates such as the widely used daphnids may also be incorporated into on-line systems. The measurand is usually the swimming activity of the daphnids assessed by using an infra-red source and receptors detecting reduced or increased movement resulting from a change in water conditions [99,117]. Such systems are currently used at the Eijsden field monitoring station on the Maas River (The Netherlands).

Other invertebrates used are bivalve molluscs such as the freshwater zebra mussel (*Dreissena*) or the marine blue mussel (*Mytilus edulis*) [118]. While measurements based on respiration, pumping and heart rates have been tested [105], valve closure or movement responses are defence mechanisms used by bivalves to avoid stress such as contaminated water [119,120]. An example of such a system is the Mosselmonitor®, which uses freshwater or marine species and may be used in continuous or in situ monitoring modes [105,119,120]. Tests using *Tubificids* worms based on behavioural changes have also been undertaken but have not as yet yielded a standardised system [121].

The exploitation of BEWS would not be successful without the elaboration of a networked scheme for data treatment and coordination of response measures to pollution events in order to mitigate their environmental impact [114]. This has been

achieved in recent years with the improvement in data transfer, personal computers, complemented by the use of on-line chemical monitoring systems (e.g., SAMOS). Requirements for a BEWS are a reliable and reproducible sensitivity to a wide range of contaminants. Calibration of BEWS with known concentrations of contaminant mixtures by obtaining dose–response relationship curves may be possible for specific applications such as at remediation sites when the contamination source is known [104]. In addition, the operation of BEWS needs to be affordable, reliable, with minimal maintenance and operational requirements, i.e. low-skilled operators requiring little training and capable of being deployed in remote or relatively unsecured sites. When operating BEWS, it is crucial to verify its sensitivity against specific target concentrations, and to achieve a high sensitivity with minimal false positives and also avoiding false negatives [99]. While their usefulness is not in question, BEWS may, however, suffer from the influence of environmental pathogens present in water [122], remain unable to detect chronic toxicity due to long-term exposure to low-level of contaminants [99] and their validation may be difficult. Acclimatisation of test organisms to contaminants present in water resulting in underestimation of toxicity [123] may be prevented by the regular change of test organisms. A further consideration is that the use of higher organisms such as fish as bio-indicators may be strongly constrained on legal and ethical grounds in certain member states.

4. Chemical monitoring

Chemical monitoring has generally relied on the use of batch or bottle sampling and chemical analysis using chromatographic and spectroscopic methods. The limitations associated with this technique have been discussed previously. This section aims to highlight a number of existing or emerging sampling and analytical tools that may be used to complement standard spot sampling.

4.1. Passive samplers

The determination of time-weighted average (TWA) concentrations, which is a fundamental part of an ecological risk assessment for chemical stressors, may be impossible without extensive repetitive spot sampling.

There are some methods that attempt to overcome the problems associated with spot sampling e.g. on-line continuous monitoring, biomonitoring and passive sampling [22]. Among these methods, passive sampling technology has the potential to become a reliable, robust, and cost-effective tool that could be used in monitoring programmes across Europe [124,125]. These devices are now being considered as part of an emerging strategy for monitoring a range of priority pollutants.

In passive sampling, a reference (or receiving) phase is exposed to the water phase, without aiming to quantitatively extract the dissolved contaminants. All passive sampling devices absorb/adsorb pollutants from water as shown in Fig. 4. The exchange kinetics between sampler and water can be described

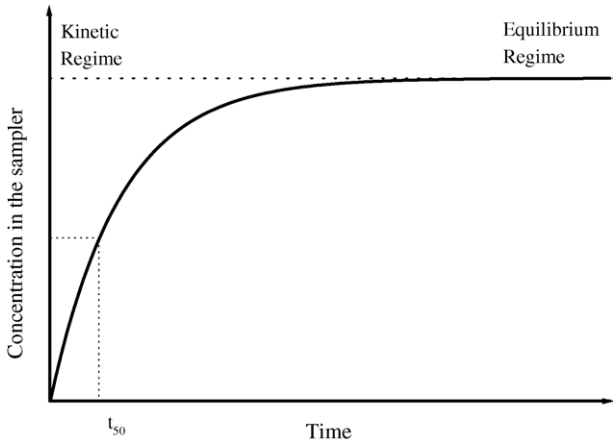

Fig. 4. Passive sampling devices operate in two main regimes: kinetic and equilibrium.

by a first-order one-compartment model:

$$C_S(t) = C_W \frac{k_1}{k_2}(1 - e^{-k_2 t}) \quad (1)$$

where $C_S(t)$ is the concentration of the contaminant in the sampler as a function of time, t, C_W is the contaminant concentration in the aqueous environment, and k_1 and k_2 are the uptake rate and the offload rate constants, respectively. Two main regimes (kinetic and equilibrium) can be distinguished in the operation of a sampler during field deployment.

In the case of equilibrium sampling, the deployment time is sufficiently long to permit the establishment of thermodynamic equilibrium between the water and the reference phase. Knowledge of reference phase-water partition coefficients allows calculation of the dissolved contaminant concentration. A review of the use of equilibrium passive sampling devices has been recently published [126]. The basic requirements for the equilibrium sampling approach are that stable concentrations are reached after a known response time, the sampler capacity is kept well below that of the sample to avoid depletion during extraction and the device response time needs to be shorter than the fluctuations in pollutant concentration being measured. Equilibrium sampling devices based on the solid-phase microextraction (SPME) principle [127] have been used to measure dissolved concentrations of pollutants in sediment porewaters [128,129] and to estimate the bioaccumulation potential in effluents and surface waters [130,131]. Passive diffusion bag samplers have been employed to monitor volatile organic compounds in water [132,133].

With kinetic sampling, it is assumed that the rate of mass transfer to the reference phase is linearly proportional to the difference in chemical activity of the contaminant between the water phase and the reference phase. When the proportionality constant or sampling rate is known, the TWA concentration of a pollutant in the water phase can be calculated. The advantage of kinetic or integrative sampling methods is that they sequester contaminants from episodic events commonly not detected with spot sampling, can be used in situations of variable water concentrations, and permit measurement of ultra-trace, yet toxicolog-

ically relevant, contaminant concentrations over extended time periods.

A range of integrative passive sampling devices has been developed and used in recent years. A comprehensive review of the currently available passive sampling devices has been published [134]. Among the most widely used samplers are the semi-permeable membrane devices (SPMDs) for hydrophobic organic pollutants [135] and the diffusive gradients in thin films (DGTs) for metals and inorganic ions [136]. Several novel passive sampling devices suitable for monitoring a range of non-polar and polar organic chemicals, including pesticides pharmaceutical/veterinary drugs and other emerging pollutants of concern have recently been developed [137–140]. Recently developed passive samplers such as the supported liquid membrane (SLM) may provide useful information on metal speciation [141]. Attempts have been made towards sampler miniaturisation combined with solventless sample processing [16]. More research is underway to develop, evaluate and calibrate a flexible device, the Chemcatcher, suitable for monitoring a broad range of priority and emerging contaminant classes including pesticides, polybrominated flame retardants, alkylphenols, drugs, mercury and organometallic compounds [142].

Results obtained with passive samplers can be interpreted at different levels of complexity. The most basic modelling concerns the comparison of peak patterns in biota and passive samplers [143,144], or between passive samplers exposed at different locations [145,146]. Samplers can be applied to investigate temporal trends in levels of waterborne contaminants [147,148] and to evaluate the location of point and diffusive contaminant sources [149–151].

In more complex applications, exposure concentrations in the field can be determined after the passive sampling exchange kinetics have been measured in the laboratory using known exposure concentrations [140,152–155]. In order to predict TWA water concentrations of contaminants from levels accumulated in passive samplers, extensive calibration studies are necessary to characterise the uptake of chemicals into a passive sampler. Uptake of chemicals depends upon their physico-chemical properties, but also upon the sampler design and is influenced by environmental variables such as temperature, flow rate, turbulence and bio-fouling of the sampler surface [156,157]. Booij et al. [158,159] described a method for estimating the uptake kinetics in both laboratory and field situations by spiking the passive sampling devices, prior to exposure, with a number of "performance reference compounds" that do not occur in the environment. The release rate of these compounds is a measure of the exchange kinetics between the sampler and water.

The (pre-concentrated) extracts obtained from passive sampling devices (particularly those used to measure organic pollutants) can subsequently be used with a variety of different bioassay procedures to assess both the level and biological effects of water contaminants [145,160]. In certain in vitro bioassays, used to assess the health of an ecosystem, problems can occur due to the difficulty of obtaining suitable water samples for testing. For example, most hydrophobic organic contaminants are present in the aquatic environment at only trace levels (i.e. $<1\ \mu g\ L^{-1}$). The extraction of several litres of water would be required to yield sufficient amounts of analyte for subsequent bioassay. Using the "bio-mimetically" sequestered extracts from passive samplers can overcome this problem [161–163]. It has also been shown that the baseline toxicity of chemicals can be predicted (based on total body residue estimates) from the concentration of pollutants sequestered by passive samplers [164,165]. The marriage of passive samplers and biomarker/bio-indicator tests offers many avenues of investigation to provide information concerning the relative toxicological significance of waterborne pollutants.

4.2. *On-line, in situ and laboratory-based sensors and biosensors*

Another set of tools that has become available to environmental managers and those in charge of monitoring programmes is sensors. There has been extensive support and collaboration for the development of these devices within Europe [166,167]. Generally three broad classifications of type are recognised: biological, electrochemical and optical sensors [168–171]. These devices are usually low cost and can be used either in situ or on-line for the rapid assessment of contamination [172]. Several sensing systems are now commercially available or at the advanced prototype stage [170,173–175].

Most of the technologies described below (Table 4) rely on a biological, chemical, or physical receptor allowing specific recognition of the chemical under study connected to a transducing element transforming the signal from the receptor into a visible and quantifiable output signal [168,176,177]. Many reviews of the different types of sensor have recently been published [170], therefore the following section will provide only examples of the many possibilities and recent technological advances offered by sensors and biosensors and will consider how they may fit within a monitoring framework.

Generally, the efficiency of a sensor may be determined by the integration or immobilisation of an adequately sensitive/selective receptor onto the surface of the transducing element and detection system [170,178]. Many approaches have been used to generate receptors based on various materials and mechanisms, and to combine them with transducers. Much work has been conducted recently with the aim of developing electrochemical and electroanalytical techniques for detection and quantitation of chemical pollutants [171]. Stripping voltammetry has greatly evolved with the development and use of modified alternative electrodes for improved detection limits, selectivity and sensitivity while avoiding the use of mercury electrodes and their associated practical complications (oxygen removal or cell cleaning) [179]. Electrochemical measurements have been miniaturised into screen-printed electrodes that are incorporated in hand-held equipment that may be used for on-site rapid on-site monitoring of many heavy metals and certain pesticides [173,174,180,181]. Molecular-imprinted polymers (MIPs) enable specific molecular recognition at their surface (similar to antibodies) and offer high stability [177] as reviewed by Yano and Karube [182]. Recognition sites are created by moulding the polymer material around a template molecule [183]. Once the molecule is removed, the material retains its shape allowing the

Table 4
Examples of the main types of commercially available and prototype sensors and biosensors that may be used for measuring organic and inorganic pollutants and monitoring of general, cyto-toxicity and geno-toxicity

Device	Recognition element	Transducing element	Characteristics/applicability	Reference
Sensors				
Electrochemical sensing	Screen-printed electrodes, cellulose-derivative mercury coated graphite screen-printed electrode	Anodic stripping (square wave) voltammetry	Heavy metals, on-site monitoring	[174,181]
	Bismuth-coated glassy-carbon electrode	Adsorptive stripping voltammetry	Chromium(VI)	[247]
	Carbon-fibre based detector	Voltammetry	2,4,6-trinitrotoluene, continuous monitoring	[248]
	PVC-based membrane + anion extruder ion-selective electrode	Direct potentiometry	Zn^{2+}	[249]
	Supercoiled DNA-modified mercury electrode	Voltammetry	DNA damage	[250]
	Single-stranded oligonucleotides immobilised onto graphite screen-printed electrodes	Chronopotentiometric stripping analysis	DNA hybridisation and detection of compounds binding to DNA and on-site measurements	[251]
	Immobilised tyrosinase hydrogel-based graphite electrode	Amperometry	Phenol	[252]
	Immunosensing film (redox polymer) on glassy carbon electrode	Amperometry	Atrazine	[252]
	Organophosphorus hydroxylase enzyme	pH-sensitive capacitive sensor chip	Organophosphate pesticides	[253]
Optical sensing	Silver-colloids embedded sol–gel substrate	Surface-enhanced Raman scattering spectroscopy	PAHs, continuous monitoring (flow-cell)	[254]
	C_{18}-silica gel beads solid surface	Solid-surface fluorescence spectroscopy	Fuberidazole, carbaryl and benomyl, carbendazim, Al(III). Continuous flow monitoring	[255–257]
	Adsorptive polymer film	Fluorescence spectroscopy	PAHs	[258]
	Chalcogenide optical fibres. Mercury–cadmium–telluride detector	Optical fibres-based infra-red spectroscopy	VOCs. In situ	[259]
	Non-ionic resin (Amberlite XAD-4) solid support	Fluorescence spectroscopy	Benzo[*a*]pyrene. Flow-through cell	[260]
Biosensors				
Cell bioassay	EROD induction in rainbow trout (*Oncorhynchus mykiss*) liver cell line	Fluorescence measurement using carboxyfluorescein diacetate acetoxymethyl ester, as indicator dye	Benzo[*a*]pyrene, TCDD and dioxin-like compounds	[261]
Yeast Environmental Toxicity Indicator (YETI)	Flurorescent gene-modified *Saccharomyces cerevisiae* + cell density	Fluorescence measurement of gene expression when repairing DNA damage	Geno-toxicity, cyto-toxicity, on-site monitoring	[186,198]
CellSense®. Mediated whole cell sensor	*Escherichia coli* immobilised onto screen-printed carbon electrodes	Current measurement based on the whole cell electron transport chain	Toxicity, on-site monitoring	[198]
SOS-*LUX*- and LAC-*FLUORO*-tests	Genetically modified *Salmonella typhimurium* TA1535 bacteria	Luminescence and fluorescence measurements	Cyto-toxicity and geno-toxicity of heavy metals	[190]
	Cellobiose dehydrogenase and quinoprotein-dependent glucose dehydrogenase enzyme-modified graphite electrodes	Amperometry	Phenols (catechol), on-site	[197]
RIANA AWACSS	Immunoassay adsorptive process	Fluorescence measurement	Pesticides, endocrine disrupters, pharmaceuticals, flow injection analysis	[194,195]
Whole cell biosensor	Genetically modified bacterial cells using reporter/promoter genes	Pollutant induced cell luminescence measurement	Phenols, PAHs, hydrocarbons, mercury, arsenic, herbicides	[96,199,262,263]

selective binding of molecules of a similar structure to the template molecule. Detection of a response may be achieved using capacitance, conductance, potentiometric or voltammetric measurements [176]. Optical measurements such as fluorescence, based on competitive binding at recognition sites between a fluorescent reporter and the analyte for the binding sites, are possible [177,184].

There is a grey area between sensors and biosensors, however, the term *bio*sensor is generally used to describe sensors incorporating an immunochemical, enzymatic, non-enzymatic mechanism, or using DNA and whole-organisms as recognition event [169,170,185]. Despite using a biological mechanism to detect and subsequently quantify contaminant levels, many biosensors have little relevance to biological functions or to organisms in a water body. However, those based on the use of whole-organisms or DNA may provide useful additional information on the bioavailability/bioaccessibility of the pollutants or on the general, cyto-toxicity, geno-toxicity, and mutagenicity of pollutant mixtures [186–191].

Some biosensors relying on immunoassay techniques have been combined with optical sensing systems and flow injection analysis for the detection of many pesticides such as isoproturon, antibiotics and endocrine disrupting chemicals [192–195]. Continuous monitoring through surface regeneration allows the possibility (separated by regeneration cycles) of over 400 measurements. While very low detection limits (ng L^{-1}) may be achieved with minimal sample preparation [194], the water sample still requires filtration prior to analysis [192]. For the Automated Water Analyser Computer Supported System (AWACSS) or the River Analyser (RIANA) systems, fluorescent-marker labelled-antibodies are added to the water sample allowing binding with the analyte of interest. Remaining free antibodies subsequently attach to analyte derivatives at the surface of the transducer. Finally, the surface is excited using a laser beam and fluorescence from the surface is detected and quantified. Thus, high analyte concentrations give rise to low fluorescence output and vice versa. The system has been designed to handle up to 32 different analytes simultaneously [192].

Enzyme-based biosensors have been developed for the field testing of river and drinking water samples or samples from waste-water treatment plants [196,197]. For example portable amperometric biosensors using two enzymes, cellobiose dehydrogenase and quinoprotein-dependent glucose dehydrogenase, have been used to analyse catechol [197]. Phenols present in the samples were first oxidised at a suitable electrical potential into a quinoid-type compound which subsequently acts as an electron–proton acceptor to react with the reduced form of the enzyme.

Research in the field of whole-cell biosensors has led to many systems which may be used to quantify general, cyto-toxicity and geno-toxicity [188,190]. Bacterial or yeast cells may be immobilised onto screen-printed electrodes (e.g. the CellSense® biosensor), in solution or added to the sample with measurement undertaken by fluorescence or luminescence [186,198,199]. Biological oxygen demand measurement may be conducted using bacterial cells immobilised at the surface of disposable sensor tips used in a three-electrode portable device [200]. Genetically modified cells include a fluorescent or luminescent reporter gene, allowing detection and quantifying of events such as DNA damage repair in the cell, pollutant catabolism, or a reduction in cell metabolism [96,103,186,190]. Test endpoint may be toxicity but certain whole-cell *bio*sensors may also be used to quantify specific pollutant levels [96,199].

Many of these systems have been developed for use as continuous monitoring systems and can provide easy, rapid (results from seconds to minutes) on-site or in situ measurements. As such they can be used for monitoring drinking water intakes, effluent discharges, the efficiency of wastewater treatment works, and surface and ground waters [201–203]. They may also be useful for mapping of contamination when it is important to obtain rapid in field results such as after accidental spills or pollution events.

4.3. Immunoassays

Immunoassay (IA) technology uses antibodies with a highly specific recognition site in their molecular structure allowing specific binding with respective antigens [204]. Recent progress has been made in the development of IAs, enabled by new strategies for the production of haptens and their subsequent attachment to carrier proteins. These developments have led to the production of antibodies based on small molecules such as pesticides which otherwise would be unable to produce an immune response [204,205]. The basic principle of most IAs is based on the interaction and binding of antigen and antibodies usually immobilised on a surface/support. The measurement generally reflects the availability of binding sites after contact with the sample containing the antigen/analyte. In order to obtain a measurable signal, a label/tracer based on fluorescence, chemiluminescence, enzymes or radioisotopes needs to be added to quantify available sites [204,206]. Therefore the quantitative measurement made using IAs is not a direct analyte concentration but may be expressed as analyte equivalents. Commonly used for small molecules such as pesticides, competitive IAs rely on the measurement of available or unoccupied sites when using a limited amount of antibodies. When free analytes and labelled antigens have been removed, the level of antibody/labelled antigens can be determined [204,207]. Similarly indirect competitive IAs involve competition between immobilised antigens and free sample analytes with free antibodies [208,209]. Once unbound antibodies and analytes are removed, labelled-antibodies are added to quantify bound-antibodies. Non-competitive IAs are based on the measurement of immobilised antibodies bound to the analytes. A second labelled-antibody reacting with a secondary site on the analyte is used for quantitation. Non-competitive IAs are not often used for relatively small molecular weight molecules such as pesticides. Enzyme-linked immunosorbent assays (ELISAs) based on the use of labelled enzyme conjugates are widely available in various designs such as coated-tubes, magnetic particles, or 96-well plates, enabling processing of a large number of samples simultaneously [204,210,211]. Enzyme conjugates are competitively displaced from binding sites by the free analytes. Tubes, magnetic particles or well-plates are rinsed and a chromogen is added

to react with enzyme conjugates producing a coloured chemical. After a period of time the reaction is stopped allowing spectrophotometric quantitation of immobilised enzyme conjugates, and thus, by difference the concentration of analytes initially present in the sample.

Quantitation is undertaken by running in parallel a set of standard solutions of known enzyme conjugate concentration [211]. Dose–response calibration curves based on the measurement of absorbance against Log concentrations are elaborated. Usually sigmoidal in shape, they exhibit a linear portion close to the IC_{50} (the enzyme conjugate concentration resulting in 50% decrease in absorbance) [204]. Other important points on the curve are the limits of detection and quantitation that determine the working range of the test. Limits of detection and quantitation for the most sensitive tests are in the ng L^{-1} range while upper limits may vary between 10 and 100 μg L^{-1} [206]. Many assays incorporate environmental quality standards (EQS) within their working ranges which render them particularly useful for screening purposes [209,212]. In competitive IAs, sensitivity is strongly linked to the difference in affinity of the enzyme conjugate or the analyte with the antibody, i.e. the ability of the analyte to displace the binding equilibrium between the antigen and the antibody. The easier it is for the free analyte to displace the equilibrium, the lower the detection limit and the more sensitive the assay [213,214]. A number of studies comparing results from IAs with those obtained by standard chromatographic techniques have shown their suitability as a low or lower cost alternative for chemical monitoring [215–217].

The specificity of IAs is greatly dependent on the extent of cross-reactivity of the assay with molecules structurally similar to the target analyte [204,218]. Certain commercially available IAs, e.g. for atrazine, present high cross-reactivity with closely related analogues, e.g. the triazine herbicides ametryn, propazine and the atrazine degradation product de-ethylatrazine, and this needs to be considered during data interpretation. However, this effect may become useful when screening broad classes of compounds [207]. Cross-reactivity can be characterised by the ratio of concentration of the analyte and the reactant [204]. However, cross-reactivity ratios may change over the working range when the dose–response curves for the analyte and cross-reactant are not parallel. Tests using different antibodies may then be chosen according to their cross-reactivity and sampling site characteristics. Assays specific to certain hydroxylated atrazine metabolites have recently been developed [208] and may offer additional information over the use of atrazine IAs on their own.

The major advantage of IA test kits is in their relative ease of use compared with chromatographic methods and they often provide comparable results [219]. Generally they are low cost, rapid and require minimal sample manipulation [216]. However, when using IAs the possible effects of environmental factors on the results must also be considered [220]. As IAs may be affected by the sample matrix (i.e. DOC, pH, and ionic strength of the water), a working range of optimum conditions is generally required [208,221]. High DOC concentrations may result in false positives by interacting with the antibodies; in these cases samples may need filtration or extraction on SPE cartridges prior to analysis [222]. While in many cases, pH does not affect IAs [204], the efficiency of IAs for chemicals with dissociation constants may decrease at pH values close to their pK_A or pK_B. In order to minimise these effects calibration standards must be prepared using water with characteristics equivalent to that of the sample. IAs have been proposed for the measurement of certain metals such as cadmium [223]. However, as only Cd(II)–EDTA complexes are being measured by the IA, samples need dilution with an excess of EDTA. Very few matrix effects were observed with a number of cations usually present in water and cross-reactivity was shown only for mercury at high concentrations [223]. Other formats for immunoassays are dipsticks [204,224], on-line automated systems [193,225] or involve the use of liposome-amplified techniques [204]. Recent developments also include express assays with the use of polyelectrolytes as carriers to reduce assay time [226] and the preparation of solid-phase immobilised tripod for fluorescent renewable immunoassay [227].

IAs are best suited for the rapid low cost screening of water samples for one particular analyte [228]. Usually no preconcentration step is needed and low detection limits can be achieved for most compounds. However, it may remain difficult to use IAs for regulatory analysis owing to cross-reactivity and analyte-equivalency issues. A negative result with IAs may easily be interpreted, whilst positive answers may require further non-immunochemical assessment. In some situations, IAs could be used to replace spot sampling campaigns providing a framework is in place to ensure the confirmation analysis of positive samples. Rapid mapping of contamination and the identification of contamination point sources are niche applications [228,229].

5. Quality assurance and method validation issues

In order to ensure the efficiency and harmonisation of future monitoring programmes, the reliability and comparability of results across member states is essential. Quality assurance (QA) and method validation schemes are crucial components in environmental sampling and analysis programmes [230]. First, a few key points need to be considered. The notion of traceability expresses ‘the property of the result of a measurement or the value of a standard whereby it can be related to stated references through an unbroken chain of comparisons all having stated uncertainties’ [231,232]. Traceability involves the use of documented standardised procedures, reference methods, the use of SI units, and reference materials (RM). An unbroken chain of comparison implies that the information contained in the sample is preserved throughout all steps of environmental analysis from sample collection to the final analytical determination. The uncertainty of each step of the procedure needs to be accounted for when assessing the uncertainty associated with the final results [232–236].

To guarantee this traceability of measurements by a laboratory involved in environmental monitoring, QA measures or infrastructures typically exhibiting four different levels are required [237]. The first level of compliance is method validation. This is, according to International Organization for Standardization, the ‘confirmation by examination and provi-

sion of objective evidence that the particular requirements of a specified intended use are fulfilled' [231] within an internal quality control scheme, based on the use of RMs, standard methods, or control charts, representing the second level of compliance. The third level relies on proficiency testing schemes to compare results from participating laboratories for the analysis of RMs. The last step is institutional accreditation [237].

Method validation may be different according to whether a method is empirical or not, i.e. whether the result obtained is dependent on the method used or not. A number of parameters are typically used for the validation of methods and include trueness, precision, selectivity, specificity, linearity, operating range, recovery, limits of detection or quantitation, sensitivity, ruggedness, robustness and, applicability, leading to the measurement of uncertainty. Misuse of terms such as repeatability, reproducibility or accuracy is common. Clear and concise definitions of all these terms may be found in Taverniers et al. [237].

For water quality monitoring, a modular approach may be useful to assess the level of uncertainty associated with each step of a measurement, e.g. the sampling stage, transport and storage, sample preparation and extraction, and the final analytical determination. Table 5 compares attributed factors of uncertainty for standard spot sampling/chemical analysis with those for the emerging tools for each step of the monitoring procedure from sampling through to the final analytical measurement. In addition, Table 5 provides an indication of how representative the data provided by the various techniques are of the biological and chemical quality of a water body. This representativeness is an important consideration during method selection. Much attention has been given to QA of laboratory-based analytical procedures in recent years [4]. Sample collection or manipulation is often neglected [230,235,238,239], yet this step remains a crucial component of the whole procedure. It is pointless to determine, often at high cost, the uncertainty of a laboratory-based analytical method if the uncertainty associated with the sampling step is high or more often unknown. Once all levels of uncertainty have been assessed, the next challenge is whether the sample collected is truly representative (over time, space and bioavailability) of the chemical conditions prevailing in the water body. As discussed in Section 2, physico-chemical characteristics of water bodies change continuously, and this can lead to variability between results obtained from batch sampling and continuous monitoring. Generally, when selecting a monitoring tool, it is important to weigh the level of uncertainty of the procedure against the representativeness of the result obtained (Table 5) for objective and unbiased data interpretation [235,240]. For example the frequency and spatial coverage that would be required to obtain a level of representativeness comparable with that using passive samplers would result in very high cost when using standard spot sampling followed by chromatographic analysis.

Many of the different tools described in this review have not yet been subjected to full method validation. A number of whole-organism bioassays such as daphnid and algal or Microtox® tests have been standardised (at the CEN and ISO levels) and their results are generally considered valid if test protocols have been closely respected. Furthermore, proficiency testing

Table 5
Comparison of types of tools from the toolbox in terms of their ability to provide a representative picture of biological or chemical conditions of a water body, and suggested level of uncertainty associated with the various modules of validation for each procedure

Modular validation approach	Spot sample + chemical analysis	Continuous chemical monitoring	Passive samplers	Bioassays	BEWS	Biomarkers	Immunoassays	Biosensors	Sensors
Sampling	Batch	Continuous	TWA	Batch	Continuous	Batch	Batch	Batch/continuous	Batch/continuous
Representativeness									
Biological quality	–	–	–	Medium	Medium/High	High	–	Low/Medium	–
Chemical quality	Very low	High	Very high	–	–	–/High	Medium	Medium/High	Medium/High
Factor of uncertainty									
Sample collection	Low	Low/Medium[a]	High[a]	Medium	Very low	Medium[a]	Low	Low/High	Low/High
Storage and transport	Low	–	Low	Medium	–	Low/Medium[a]	–	Low/–	Low/–
Sample preparation	Low	Low	Low	Low	–	Low/Medium	Medium	Low/Medium	Low/Medium
Sample extraction	Low	Low	Low	–	–	Low/Medium	–/Low	–	–
Analytical determination	Low	Low	Low	Low	Medium/High[a]	Low	Medium[a]	Low/Medium	Low/Medium

Key: TWA: time-weighted average concentrations.
[a] May exhibit difficulties in the validation step.

schemes and inter-laboratory trials may be conducted relatively easily.

Pre-validation can assess the scope of validation prior to full validation [237]. The scope of validation is a crucial stage for emerging technologies as the same results are not necessarily expected from tools within sets of similar techniques. Therefore, applying a validation approach based on the assessment of trueness, precision, selectivity, specificity, limits of detection and quantitation, working range, ruggedness and robustness or sensitivity may not be appropriate. In addition to a lack of availability of (and need for) RM mimicking surface waters and other matrices based on sediment and biota for priority substances [4,241,242], their use may not be practical for certain types of emerging tools. For example, the calibration of passive sampling devices using (certified) RMs would require significant (several hundreds of litres) volumes of material. As standard RM volumes are generally no more than 1–2 L this would not be practical or cost effective [243,244]. In addition, it may be difficult to produce and store RMs for emerging technologies for which the measurand is a specific fraction of the pollutant present in the water (owing to metal speciation or pollutant bioavailability). For example, assessing the accuracy or trueness of determinations made by passive samplers may prove difficult, as the results obtained may not be directly comparable to total concentrations found using spot samples or filtered samples [245]. However, improvements in calibration of passive devices may be achieved by using pre-loaded performance reference compounds [159].

The validation process should reflect the use of a particular technique, e.g. it is not expected that a BEWS would have a specific operating range. However, it is crucial that a positive result (i.e. an alarm) is obtained *via* this system for specific toxic conditions, while reducing false positives by minimising the effects of other parameters not strictly related to chemical toxicity [99]. It may also be difficult to use IAs for quantitative measurements especially in the case of regulatory analysis owing to cross-reactivity and the analyte-equivalency unit issues. Full validation would require extensive cross-reactivity testing which is impractical. New approaches to the validation process may be required to enable an efficient use of emerging techniques within the WFD.

6. Conclusions

In light of evidence presented in this review, the successful implementation of the WFD will rely on a number of factors. Standard (classical) monitoring based on spot sampling and chemical analysis can be used but has severe limitations for certain types of monitoring. Many of the emerging tools and techniques that have been developed in recent years provide suitable alternatives for low cost and more representative monitoring. They can provide additional information on temporal and spatial variability of pollutants, biological/toxicological effects, account for contaminant bioavailability, as well as early detection of pollution events. While the choice of a suite of tools for a specific monitoring task will be critical, this selection will depend on the type of information required and the cost of tools. However, one needs to take into consideration that all these tools measure different fractions or have different endpoints/outputs (even within one class of tool). A clear understanding of the significance of the results obtained with these techniques is essential, particularly when comparing these with historical data that may have been gathered using other methods. Therefore, the successful application of the tools included in an environmental manager's toolbox will require a clear understanding of what exactly is being measured in the field. All the techniques need to follow unambiguous protocols for each part of the monitoring and analytical steps. Quality assurance structures must be set up to allow efficient and harmonious monitoring across Europe, and ensure reliability and comparability of data. Due to the nature of the technologies themselves, and the complexity of the system under study, it may prove difficult to obtain accreditation for certain tools and hence to use them for compliance checking and other legislative purposes. In this context, another priority issue for a successful inclusion of emerging techniques in water monitoring programs is to improve communication between scientists and policy-makers, and to optimise the coordination between scientific development outputs and policy-research needs [246].

However, there is little doubt that the combination of these technologies, together with associated ecological monitoring, should enable the representative assessment of the health of an ecosystem, as required by the WFD.

Acknowledgements

We acknowledge financial support from the European Union's Sixth Framework Programme (SWIFT-WFD, contract no. SSPI-CT-2003-502492) and technical assistance from all the SWIFT partners who were involved in the production of the directory.

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