Water and Soil Monitoring for the Protection of Environment and Human Health

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ABSTRACT

The first pillar of the protection of the environment and also, as a positive consequence, of the human community living in this environment, is the establishment of protective monitoring programs. Current monitoring programs for water and soil are based on sampling and laboratory analysis of chemical and microbiological variables. Parallel to this traditional approach, methods to measure effects directly on living organisms, at both individual and community level, have been integrated into monitoring plans. The present paper reviews the state of the art of the research activities in the field of water and soil monitoring carried out by the Italian National Research Council (CNR) Institutes: this review is the outcome of a survey conducted by the Working Group 2 established in the framework of the CNR Environment and Health Inter-departmental Project , PIAS CNR. Emerging problems, such as the presence of nanoparticles and perfluorinated compounds in the environment, and future developments of the monitoring techniques are also discussed.

1. Introduction

The protection of the environment and human health from toxic agents is traditionally based on the selection of a list of potentially dangerous substances or agents and the statement of the corresponding emission limit values or quality standards.

Methodologies to establish quality standards are based on physicochemical and toxicological data which are normally collected and organized in a risk assessment document. The prioritization procedure used to establish the list of pollutants is based on the knowledge of the toxicological properties and data on use and production amounts. In order to be validated, all these procedures need a large amount of data which are actually not available for the millions of synthetic molecules prepared and brought into the market.

As a consequence, the approach, based on emission limit values at the discharge and on environmental quality standards in the receptor compartment, could not be really protective for the environment.

A step forward in the legislation on water quality protection was the publication of the Water Framework Directive (WFD, Directive 2000/60/EC) that introduced the concept of water bodies protection: it moved from a regulation based on emission control to one that is based on the protection of the ecological quality of receiving water bodies. On the assumption that the repression of discharge by imposing emission limit is not sufficient to protect the environment, it is crucial to verify whether the receiving body is able to support activities which are imposed on it, keeping as much intact as possible the ecological community that resides there. The aim of the WFD is to achieve a good quality status from the ecological point of view, namely to ensure that all bodies

could support a biodiverse ecological community.
In parallel with this innovative approach based on ecological classification, in order to control the chemical pollution, WFD establishes a priority list of compounds which, for production volumes and/or use and hazardous characteristics in terms of toxicity, persistence and bioaccumulation, pose a risk to the aquatic ecosystem or to human health. The pollution control is based on a combined approach which sets limits on emissions (left to Member States) and maximum allowable levels in the receiving water body, expressed as environmental quality standards (EQS) which are fixed in a recently adopted EC Directive (105/2008/EC).
The use of living organisms and their community as monitoring tools has many advantages. Organisms, living in the environment under study, are constantly exposed to the physical, biological and chemical influences of that environment. Organisms can often accumulate significant quantities of compounds even if exposed to very low concentrations in the environment.
It is nevertheless difficult to correlate the measured adverse effects on the ecological community with the presence of specific classes of chemical compounds because also the hydromorphological and physicochemical alteration of the natural habitat influences the structure of the community. In order to detect sub-lethal effects, single living organisms are the best indicators of environmental alteration: if chemical tests only detect "known" substances, the measuring of effects on organisms by appropriate biomarkers can highlight not only the biological response to unknown substances but can also evidence the synergistic effects that may be caused by a mix of different substances.
Integrated monitoring systems are the most effective tool to highlight the interactions among substances by pointing out the responses at different levels: responses which can be so lethal that they affect the composition of the community, or sub -lethal responses that act on the body-metabolic-physiological models or interact with genetic transmission mechanisms.
Low-cost or highly innovative technologies have been developed for the application in field/in situ (spectroscopic or sensing), in order to achieve a rapid characterization of the site with a high spatial/temporal resolution; in alternative to chemical or physicochemical monitoring systems, techniques based on the measurement of biological response have been proposed, as they can evaluate the possible risk, even in the absence of a direct chemical measurement of a particular pollutant. The role of biomarkers, possibly integrated in a biosensor system, is crucial for the development of an early warning system which could prevent adverse effects on the ecological community and human health.

2. Working methodology in working group 2 of the CNR Environment and Health Inter-Departmental Project (PIAS)

The working group 2 (WG2) on "Monitoring Systems for Soil and Water", established in the framework of the PIAS-CNR Project, has defined its field of interest in the development and use of monitoring techniques, technologies or innovative methods for soil and water monitoring, where a situation of environmental pollution represents a potential risk for human health.
After a preliminary review of literature focused on the various techniques developed for the monitoring of soils and waters, WG2 researchers were invited

to put together their scientific expertise and produce a state of the art report of the activities regarding the monitoring of environmental impacts which could be relevant for human health. Assuming the central role of the integrated approach, a review of the different professional profiles present in the CNR Institutes was carried out, to facilitate the creation of a multidisciplinary group of researchers and the sharing of different expertise ranging from instrumental analytical chemistry to ecological assessment.

3. State of the art of CNR activities

Ecological risk assessment (ERA) has been defined as ‘‘the practice of determining the nature and likelihood of effects of anthropogenic actions on animals, plants, and the environment’’ (1). A correct analysis of the complex interactions between the pollution caused by humans and the environment requires the application of a multidisciplinary approach and the determination of different parameters, that can describe the exposure levels and convert them into individual warning situations (2).

A clear example of this operating method is described by the Triad approach used in the ecological risk assessment of sediments (2,3) and allows the investigation of the possible negative effects of toxic chemicals at different levels of biological organization, from single organism to population and/or community level (2). The Triad paradigm enables the assessment of potentially hazardous effects on ecosystems by simultaneously considering chemical concentration, bioavailability of pollutants and the ecotoxicological profile of the environmental matrix under observation. The latter is usually determined by a set of ecotoxicological tests as well as by monitoring possible ecological alterations, quantified by changes in different structural and functional community attributes. This integrated approach on different levels of monitoring should be adopted to provide full details of the impact on both the water and the soil in the site of interest. Methods that are relevant at different levels of specificity are needed because some parameters (such as biomarkers) describe effects at suborganism levels of biological organization (4) and different phases of stress syndrome evolution in model organisms (5,6). On the contrary, other ecotoxicological endpoints (e.g. survival and reproduction) indicate possible direct effects at population level (7). Chemical analyses reveal the presence of potentially dangerous substances in soils, but cannot be used to quantify bioavailable fractions (8) that play a more relevant role in threatening ecosystem integrity (9-11). Finally, a direct evaluation of community structure and functionality should clearly detect overall environmental effects on ecosystems (12) .

As everybody knows, biomarkers have been defined as sublethal responses to environmental chemicals at different levels of biological organization (e.g, molecular, cellular, tissutal, physiological, behavioral, of organisms) which evidence an alteration respect to the natural status (13,14).

Toxic effects caused by exposure to environmental pollutants can alter endpoints at different levels of biological organization (4,15) (Fig. 1).

In particular, the classical biological tools applied in ERA (i.e, bioassays and ecological parameters) are able to highlight the impairments from the organism to the population–community level. This analytical system cannot, however, be used to investigate early effects in organisms exposed to pollutants (i.e, from early

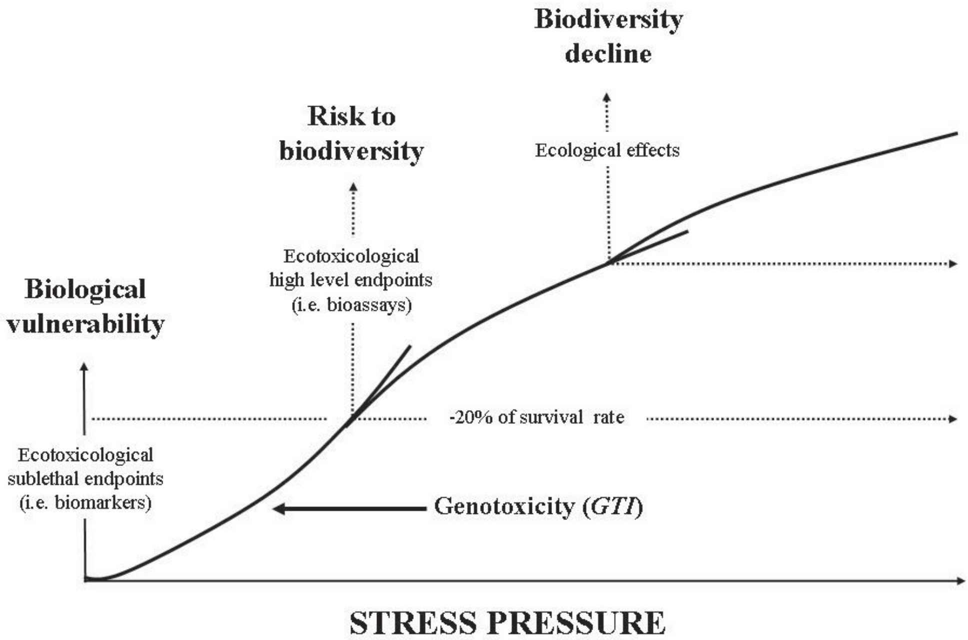

Figure 1: Different biological toxic effects induced by environmental contamination. From Dagnino et al. (15).

sublethal stress syndrome to the onset of reduced survival). The investigation of the initial phases of biological impairment plays a crucial role in determining the vulnerability level reached by the biotic resources in those cases where no evident changes in the traditional, high level endpoints are detected. Therefore, in order to complete the analysis of the spectrum of possible biological effects induced by environmental contamination, the alterations in sublethal endpoints measured on sentinel organisms can be evaluated (6). In spite of the high sensitivity of these types of parameters, it must be stressed that it is possible to clearly infer the stress syndrome degree in organisms exposed to toxic chemicals by the application of ad hoc models. This is done by using a complete battery of biomarkers at different levels of biological organization (i.e, molecule, cell, tissue, organ, organism) on model organisms (5) .

Therefore an extensive monitoring requires different skills, ranging from purely analytical capacities for the identification of compounds present in the matrix under investigation, up to bio-molecular techniques to assess effects of pollutants on the gene expression.

In the following paragraphs, a survey on the activities of CNR institutes will be presented, covering many of the skills described above and needed for the establishment of an effective monitoring project. The covered field of expertise ranges from advanced chemical analysis, in laboratory as well as field research, to traditional ecotoxicological assays, biomarker assessment in exposed and natural organisms, up to studies about the alterations of structure and function of ecological communities.

3.1 Laboratory chemical analysis

The off-site instrumental techniques are characterized by manual sample collection and transfer in centralized laboratory units where advanced analytical equipment is available. Many chemical monitoring activities with instrumental methods are currently on-going in CNR Institutes, also in response to the Water Framework Directive (WFD, 2000/60/EC) requirements.

Several groups are involved in monitoring metals in Italian soil, surface and groundwater. Metals present in soil and waters can accumulate in the trophic chain and represent a risk for the final consumers. The Tuscany region in Central Italy, due to geological and historical reasons (past mining activities present in the territory since the Etruscan era), is particularly impacted by metals and many CNR groups are working on metals contamination in this area.

Trace metal concentrations were monitored in some urban soils of three medium sized towns of coastal Tuscany. (16) Soil samples were collected in roadsides, urban agricultural soils (allotments), playgrounds and public parks. The analysis included total metal content (Pb, Cu, Zn, Ni, Cd), and sequential extraction. Lead reached the highest levels in the soils and was higher near roads. In urban agricultural soils and in allotments Cu was present in noticeable quantities (300 mg/kg). The presence of Cu in urban soils seems to be typical of soils used for a long period as agricultural land, especially vineyards in the area covered by this study. Sequential extractions were performed to evaluate the mobility of the metals and to better understand the impact of the anthropogenic activity on urban sites.

Mercury contamination in the Cecina river basin (Tuscany, Italy) has been studied by Scerbo et al. (17). Mercury was measured in waters, sediments and fish of the river and its most important tributaries. In fish samples the organometallic metabolites of mercury were also determined. Particularly high concentrations were found in the sediments of the S. Marta channel flowing into the Cecina, where a chloro-alkali plant discharges its wastes, and high levels were still detectable 31 km downstream from the confluence, bioaccumulating also in fish species.

Italy, and particularly Tuscany, is strongly interested by boron contamination because of the presence in its territory of active volcanism, geothermal activity and mineralized areas. The compliance with the EU normative is technically complex and economically very expensive. The limit of 1 mg/l imposed by the European Union for boron in drinkable waters (98/93/EC) is based on the "precautionary principle", considered that the effect of boron on humans is at present poor and contradictory.

New geochemical and isotopic investigations on waters (δD, $\delta^{18}O$, $\delta^{11}B$, $\delta^{13}C$, $\delta^{15}N$, $^{87}Sr/^{87}Sr$) and soils ($\delta^{11}B$, $^{87}Sr/^{87}Sr$) were carried out in Southern Tuscany where boron anomalies occur and the assumption of this element through drinking water or agricultural products could have an adverse effect on the health of the local population (18-21).

The determination of abnormal concentrations of trace metals in soil is crucial to highlight the possible presence of contamination. These abnormalities are identified on the basis of the knowledge of natural concentrations, expressed in terms of "background" (natural background levels) or "baseline" (currently found contents). The criteria for the determination of these "natural" concentrations have been the subject of intense international debate for many years. It is therefore necessary to evaluate the potential of other pollution indicators from diffuse sources, complementary to the existing ones (soils and sediments). In order to achieve this, the properly standardized use of higher plants offers a promising tool to establish a precise date for the event and to assess the spatial extension of the contamination (23-25).

In parallel to soil and groundwater studies, presence, distribution and accumulation of metals in sea areas were investigated: samples of *Mytilus galloprovincialis* were collected monthly during the July 1999-June 2000 period from two mussel culture areas influenced by urban and industrial wastes (26). These stations, subject to different environmental impact conditions, are located in the coastal area of Taranto Gulf (Ionian Sea, Southern Italy). Metals (Cd, Cu, Pb, Zn, Fe and As) were determined by atomic absorption spectrophotometry (AAS) in the whole soft tissue of mussels. Seasonal changes in metal concentrations were observed. Metals exhibited maximum values in later winter-early spring, followed by a progressive decrease during summer.

Metal concentrations were similar to those detected in other Italian coastal zones, and indicate that the seafood under investigation poses no hazard to human health because metal content is within the permissible range established for safe consumption by humans.

For many years the presence of toxic inorganic fibrous particles such as asbestos in drinking waters has been of great concern for their direct impact on human health.

The assessment of the diffusion of inorganic fibrous particles in the environment is performed through detection, identification and quantification of mineral inorganic particles present in animal and human tissues and biological fluids from impacted areas. In these same areas, a comparison is carried out among the types and amounts of particles detected in the biological samples and the fibers present in aerosol and mineral outcrop. An evaluation of the biological effects of some of the fibrous mineral phases present in the rocks is also performed (27-30).

In the field of the advanced methodologies for the monitoring of organic micro-pollutants in water, the CNR Water Research Institute (CNR-IRSA) plays a well acknowledged role. Besides the determination on “classical” persistent and priority organic pollutants (chlorinated pesticides, PCB, PAH, alkylphenols), advanced analytical methods based on LC-MS technique are under development for the determination of metabolites and emerging pollutants in various surface, drinking and ground waters in Italy (31-33).

Emerging substances are those compounds or groups of compounds produced or used in significant quantities but which are not currently restricted by regulation due to the lack of information about their effective environmental diffusion and toxicity. In this category, many substances are polar substances such as perfluorinated compounds, PPCP (pharmaceutical and personal care products), estrogens. The CNR-IRSA research group is also the national focal point of an international network, NORMAN (Network of Reference Laboratories for Emerging substances) and the one responsible for the substance prioritization procedures at European level under the Water Framework Directive Common Implementation Strategy.

3.2 Biomarker-bioassay

Bioassays are typically used to measure the effects of some substance on a living organism.

They can be classified on the basis of the type of response, by discriminating the end-point level: a high response level is associated to the survival rate or reproduction inhibition, while a low response level is connected to sublethal effects that are revealed by specific physiological or genomic alterations. Among the former type, the most used ecotoxicological tests are those based on the measurement of EC50 (Effect Concentration) or LC50 (Lethal Concentration), i.e. the concentrations which exert an effect on 50% of the organisms under test. The latter type of bioassays is based on the measurement of specific biomarkers: experimental tests evaluate effects that are not lethal for the organism, like a change in a metabolic protein or a behavioral modification. As indicated by Dagnino et al. (15), the separate evaluation of different levels of response helps to avoid a misinterpretation of the ecotoxicological results.

At the CNR Marine Science Institute (CNR-ISMAR), studies are carried out about the health condition assessment of marine invertebrates and vertebrates in relation to environmental stressors. (34-36) Moreover, there is a great interest about the deployment of biochemical, histo-cytochemical and histopathological biomarkers as early warning systems in coastal marine environments monitoring that is the object of the research at the CNR Institute of Biomedicine and Molecular Immunology (CNR-IBIM) Cell Stress and Environment Research Unit (37-46). This research group has contributed to the identification of cellular and molecular stress markers in the marine environment by using the sea urchin as a model system. This is a common organism in our shores and has a great ecological and commercial importance. Laboratory as well as field experiments showed that, when responding to chemical (heavy/essential metals) or physical (temperature, acidity, ionizing radiations) stressors, the adult immuno-competent cells and embryos or larvae of the sea urchin express specific markers, better known as stress proteins, including heat shock proteins (hsp70) (39-41), metallothionein, (38) and acetyl-cholinesterase (37). Other studies have shown that environmental stress causes DNA damage in the form of broken single- and double-strand (44), and variations in the levels of other stress and apoptotic markers in response to exposures to heavy/essential metals (cadmium/manganese) and/or UV-B/X radiation (42,43). Results at cellular and molecular (proteins and mRNA) levels from laboratory exposures were compared to those obtained using samples collected in field studies carried out in the Mediterranean and Northern seas, in order to bridge together field ecology and laboratory-oriented molecular toxicology (45,46). Most of the markers tested were sensitive to the stress conditions used. The results of the research support the suitability of sea urchin cells and embryos as valid tools to bio-monitor the effects of physical and chemical stress on marine aquatic ecosystems.

At the CNR Biophysics Institute (CNR-IBF) studies on the effects of environmental pollutants in eukaryotic microorganisms are ongoing; specific type of physiological, cellular and molecular level responses have been identified in the presence of environmental pollutants (47-52). This kind of studies is a valuable complement to chemical analysis, as it can provide valuable information on the potential toxicity to the organs, in order to detect the first symptoms of exposure. The effects studied include:

- the change in the intracellular pool of non-protein thiols (glutathione and phytochelatins) in phytoplankton algae

to be used as a biomarker of heavy metals bioavailability.
- the variation of photosynthetic microorganisms in aquatic and motility in response to exposure to environmental pollutants.
- short-term genotoxicity tests on cell cultures of specific strains of yeast used as a model system.

An integrated approach of biological assays performed with different microorganisms can be applied to water and sediment elutriate collected in impacted coastal and inland areas, such as estuaries, ports, urban areas.

In vivo measures by micro-spectroscopy and micro-spectrofluorimetry of the photosynthetic compartment of planktonic species, present in water contaminated by organic compounds and/or heavy metals, can determine the effects of these contaminants on the composition of pigments, the ratio chlorophyll: carotenoids, photosynthetic efficiency (53-56). Toxic algal species identification and quantification could be done using techniques of optical microscopy, and/or fluorescence for taxonomic recognition with image processing techniques.

The Venice Lagoon (Northern Italy) is an attractive area of study due to its historical interest and ecological fragility. A spatial and temporal survey at three sites located in the "canals" of Venice city centre and at a reference site in the Lagoon was undertaken to evaluate stress effects on mussels sampled in Venice urban area, where raw sewage is discharged without treatment (57). A battery of biomarkers (metallothionein, micronuclei, condition index and survival in air) was used to evaluate the stress condition of the animals. At the same time, an alkali-labile phosphate assay (ALP) was performed in mussel hemolymph to find a biomarker of the estrogenic effect for this species. Biomarker results showed an impairment of the general health condition in the mussels coming from the urban area, in agreement with the chemical analysis.

Another study (58) surveyed the water quality in Venice urban areas in connection with the discharge of untreated sewage directly into canals, in addition to the pollutant load already present in these areas. One way of estimating the impact of these chemicals is the monitoring of the local fauna. In the search for good water quality indicators in Venice urban area, two physiological indices for mussels (*Mytilus galloprovincialis*) - survival in air and condition index - have been evaluated. Native mussels and also those transplanted into the urban area showed reduced survivability in air and decreased condition index values, indicating a less healthy status in animals collected from the urban canals. Data are discussed in relation with pollutant bioaccumulation.

Coastal environments are highly variable on a daily scale. In these environments, benthic foraminifera, a class of marine Protista, can be used as bioindicator (59). These organisms can define the extent of similar environmental conditions (biotopes) through the study of the structure of their assemblage (presence – absence - relative dominance). The monitoring results show the capacity of the benthic foraminifera to monitor the changes occurring in unstable environments and to indicate the evolutionary trends of transitional environments.

The use of molecular techniques such as PCR assay (Polimerase Chain Reaction) for the determination of the genotoxic effects induced by the pollutants in the monitoring programs is rather recent.

This tool was employed in an assessment

study on recharged aquifers (60): this practice presents advantages for integrated water management in the anthropogenic cycle, namely, advanced treatment of reclaimed water and additional dilution of pollutants due to mixing with natural groundwater. Nevertheless, this practice represents a health and environmental hazard because of the presence of pathogenic microorganisms and chemical contaminants. In this study, the groundwater recharge systems in Torreele, Belgium, Sabadell, Spain, and Nardo, Italy, were investigated for fecal-contamination indicators, bacterial pathogens, and antibiotic resistance genes over the period of one year. Real-time quantitative PCR assays for *Helicobacter pylori*, *Yersinia enterocolitica*, and *Mycobacterium avium subsp. paratuberculosis*, human pathogens with long-time survival capacity in water, and for the resistance genes (ermB, mecA, blaSHV-5, ampC, tetO, and vanA) were adapted or developed for water samples characterized by different impacts. The resistance genes and pathogen concentrations were determined at five or six sampling points for each recharge system. The three aquifer recharge systems demonstrated different capacities for removal of fecal contaminants and antibiotic resistance genes.

A targeting species-specific PCR assay was combined with a filter system to collect phytoplankton cells and get spatial and temporal series as part of the Mediterranean Sea EU project Strategy (61). The application of PCR allowed a rapid detection of several harmful dinoflagellate species and genera, including *Alexandrium* spp. Field samples were concentrated on filter membranes, total DNA was extracted from mixed phytoplankton populations and PCR assays were carried out with specific primers. Qualitative PCR results were compared with light and epifluorescence microscopic examinations. Results indicated that this molecular assay was able to detect harmful target cells at concentrations undetectable by microscopy. The application of this filter PCR assay to seawater samples showed to be a sensitive and rapid procedure for the routine monitoring of coastal waters.

3.3 Ecological community studies and microbiology

The classification of the quality status of a water body through the study of the resident ecological community at different tiers is currently widely diffused in European monitoring programs also thanks to the impulse received by WFD. The effect of stressors on the ecological community is evaluated both at structure and function levels. Macrophytes, diatoms, macrobenthos and fish are the mostly used ecological quality indicators.

Among the other biological components of the ecological community in both water bodies and soils, special attention should be paid to the microbiological community for its functional role, ubiquitous presence and possible direct impact on human health.

In fact, the study on the natural capacity of bacterial communities to remove xenobiotics (pesticides, pharmaceuticals, biocides) in soil and water could have a very important implication for human health protection. The CNR-IRSA is currently studying natural bacterial communities from contaminated sites: specific bacteria strains that, after repeated exposure to xenobiotic, adapted themselves and became able to remove pollutants through metabolic and/or cometabolic processes, were isolated and identified (62-67). This “self-purification” ability can be used for “recovery strategies” (i.e. bioremediation) of contaminated sites.

The knowledge of the microbiological quality of coastal waters and marine organisms (fish, crustaceans and molluscs) (68-70), that is fundamental to assess the sanitary and ecological risk in a coastal zone, is a central research issue for the CNR Institute of Coastal Marine Environment (CNR-IAMC).

A recent work by Caruso et al. (71-75) is focused on the use of bacterial indicators to assess the anthropogenic pressures over coastal aquatic environments. Selected bacterial species (*Escherichia coli, Enterococcus* spp.) or related parameters allow to track the occurrence and evolution of bacterial pollution, and to prevent human health risks caused by the use of polluted waters.

Up-to-date standard procedures for bacterial pathogens determination and identification are necessary, due to the limitations of the usual culture methods (long response times, low specificity). In recent years, research efforts have been devoted to the improvement of technical equipment (automatic multiple samplers) and methodologies for the assessment of seawater microbiological quality particularly addressing the detection and enumeration of *Escherichia coli* or *Enterococcus* spp. in seawater as faecal pollution indicators. Rapid methods such as the fluorescent antibody method and the β-glucuronidase assay have been developed and optimized to monitor bathing waters, allowing the quantitative measurement of target bacterial molecules and accurate quantification of faecal pollution phenomena. Combined fluorescence-staining protocols have also been set up, in order to detect bacteria which may present a pathogenic potential. Data obtained by these new analytical procedures encourage the use of *E. coli* or related parameters as successful tools for early warning of seawater bacterial pollution and for the screening of polluted coastal areas; therefore they offer interesting perspectives to prevent waterborne diseases.

The microbiological quality of coastal waters is usually estimated by determination of faecal indicator bacteria. However, bacterial species possibly pathogen for humans could occur also as microorganisms indigenous to coastal marine environments (e.g. *Vibrio* spp.). Since their concentration is related to the temperature of coastal waters, and unrelated to classical faecal indicators, monitoring and control of this bacterial group is needed to plan preventive measures for human health protection. (70,76-78) The *Vibrio* genus is widespread in coastal waters and includes more than 63 species. The most well-known species is *V. cholerae*, which causes cholera epidemics worldwide. In addition to *V. cholerae*, many other *Vibrio* spp. are recognized as potentially human pathogens causing 3 major syndromes of clinical illness: gastroenteritis, wound infections, and septicemia. Epidemiologic data suggest that the majority of these infections are foodborne disease and associated with raw or undercooked shellfish. In wild and cultivate shellfish the bacterial density may reach high concentration and potential toxic effect for humans. For this bacterial group the standard microbiological criteria used to assess water quality have to be revised.

The analysis of antibiotic resistance patterns (ARPs) of faecal indicator bacteria allows to detect the presence and persistence in the environment of genes linked to antibiotic resistance. Research is in progress to characterize the ARPs of enterococci, as they are emerging pathogenic bacteria, and of *E. coli* for their capacity of acquiring antibiotic resistance and spreading their resistance to other species *such as Salmonella, Shigella, Yersinia, Vibrio etc.* (79-81).

Studies on *E. coli and enterococci* have particular epidemiological and ecological relevance because these micro-organisms can occupy multiples niches, including humans, mammals, birds, reptiles and insects.

3.4 Advanced technologies and early warning systems

Advanced technologies have been applied to the monitoring of soil and water for the protection of human and environment health.

Among those, interest is growing for the application of innovative microscopy techniques, such as scanning probe microscopy (SPM), atomic force microscopy (AFM) and scanning near-field optical (SNOM) to the environmental field. These techniques exploit the interaction of a functional tip scanned over the surface of a sample (solid or adhesive on the substrate) to reconstruct the morphology of the sample and, simultaneously, achieve super-resolution maps of other properties of the sample (i.e. local optical and fluorescence properties, maps of surface friction and nano-mechanical properties, magnetic domains, etc.). These microscopes are very useful in the study of nanostructured materials, surfaces and interfaces (i.e. sensors, biosensors, biocompatible surfaces), and analysis of nanoscale biosystems that can be investigated at the cellular level, subcellular and macromolecular aggregates. In particular, biosystems can be studied in physiological conditions and can describe temporal, three-dimensional and quantitative evolution (morphometry). (82-88) In addition to the development of innovative tools, expertise has been gained over the years in the study of intra-cells interactions in culture and exposed to environmental agents (drugs, electromagnetic fields, heavy metals, UV etc.) or nanostructured agents (nanoparticles or nanotubes) and in the study of complex phenomena such as cellular aging or apoptosis.

An important research field is the integration of advanced technologies in measuring systems that can be used for on-line or in-situ monitoring. The final goal is to make available some devices that can act as an early warning system of sudden alteration in the environmental quality status.

In the CNR-IRSA, Liquid Chromatography with Mass Spectrometric detection (LC-MS) has been integrated with an automatic sampling and preparation station into an on-line monitoring station for the determination of polar organic substances in drinking water (89). This system can be used in potable water treatment plants for the control of influent and effluent. In this way, a control on the incoming water quality and efficiency of the treatment procedure should be achieved. The operating limitations of this station are linked to the total cost of the equipment, that is still too high for a massive deployment, the reduced frequency of sampling and the need for highly qualified professionals for the frequent maintenance required.

Spectroscopic techniques are suitable to be integrated in small portable or fixed station devices for in-situ monitoring by optical fiber detection.

The concentration of several pollutants, usually present in industrial waste waters, could be predicted by the neural network data processing of absorption and fluorescence measurements in the visible spectral range. Proper network tuning provides quantitative analysis of many pollutants with sub-ppm resolution.

Compact optical fiber instrumentation for absorption spectroscopy and an innovative flowcell for fluorescence measurements enable cost-effective, in situ, nonstop monitoring of waste waters (90).

In the framework of the development of new methods for measuring and monitoring soil pollution, the use of magnetic susceptibility as a proxy variable for monitoring heavy metals in soils has been explored (91). Magnetic measurements are carried out by using a magnetic susceptibility meter with two different probes for in situ field surveys. The relationships between heavy metal levels and magnetic susceptibility values of soil samples were assessed. Results suggest that a careful check of the experimental procedure plays a crucial role for using magnetic susceptibility measurements in situ monitoring of heavy metals.

Environmental management needs some tools capable of providing, over a relatively short time, integrated responses regarding the levels of contamination and the ecological consequences on different compartments of the concerned ecosystems. The biosensor "tool", that responds precisely to this necessity, consists of a biological active element - from an isolated enzyme to a whole organism - immobilized on a transducer system (sensor) for the selective and reversible determination of the presence and/or the concentration of certain chemical molecules in a sample. In fact selectivity and sensitivity, together with the possibility to have a portable tool are the main advantages of biosensors.

A compact and portable sensing device that combines the production and detection of hydrogen peroxide in a single flow assay has been proposed for herbicide detection in water (92). The principle on which the biosensor is based is that herbicides, under illumination, can inhibit the photosystem II electron transfer. Photosynthetic membranes isolated from higher plants and photosynthetic micro-organisms, immobilized and stabilized, can serve as a biorecognition element for a biosensor. The inhibition of photosystem II causes a reduced photoinduced production of hydrogen peroxide, which can be measured by a chemiluminescence reaction with luminol and the enzyme horseradish peroxidase.

Systems that use biological responses to detect environmental quality changes in continuous (on-line monitoring) in a simple, quick and economical way can be used as Biological Early Warning System. In this field, the possible use of an electrochemical growth signal transduction of a natural biofilm (microecosystem) on a suitable metallic substrate, was recently investigated with the aim of revealing, in real time, any alteration of the normal development of the microbial community induced by the presence of toxic substances in the aquatic environment (93-95). The prototype of the innovative patented biosensor shows that the biological electrochemical signal is significantly inhibited in the short term (minutes-hours) by known concentrations of a series of reference toxics.

Among the most advanced early warning systems, based on in vivo response of organisms to toxic agents, a Swimming Behavior Recorder System - able to measure the swimming behavior of marine invertebrates larvae exposed to toxic substances and/or environmental matrix under controlled conditions - has been presented (96-99). The methodology for the detection of alteration in swimming uses a prototype system to analyze video graphics, capable of automatically recording the aquatic organisms swimming speed, and providing, on the basis of the alteration of this parameter compared with a control, two toxicological endpoints: immobilization (acute) and alteration of swimming speed (sub-lethal).

3.5 Auxiliary techniques for monitoring

Geophysical techniques have been used for several years to measure hydraulic parameters in the monitoring and control of groundwater contamination. The most common techniques are the electromagnetic ones that are more sensitive to any changes in physical parameters (i.e. electrical conductivity) of soils and sub-soils caused by the presence of a particular contaminant in the water tablet or soil porosity. Therefore, the use of geophysics monitoring systems should be able to follow over time and space areas the evolution of a particular case of pollution, such as, for example, a leakage from a dumping site. The study and application of these non-invasive and low-cost technologies, integrated with the more traditional ones (sampling and diagnostic studies), will lead to a knowledge of the land and the environment, in order to provide a better safeguard level and to plan remediation procedures.

In the Hydrogeosite laboratory of the CNR Institute of Methodologies for Environmental Analysis (CNR-IMAA) a simulation plant has been built to perform hydrogeophysical experiments for the integrated study of contaminated soils and subsoils with the aim of creating a standard methodology for practical intervention (100-102).

The activity carried out by the CNR Research Institute for Geo-hydrological Protection (CNR-IRPI) in the field of erosion and hydro-meteorological monitoring is of fundamental importance for the protection of the water bodies and their basin.

4. Emerging issues

The literature review highlights that the monitoring focus is still mainly addressed to some classes of well studied molecules, such as trace metals and persistent organic pollutants, which represent a typology of pollution that emerged several decades ago, but for which there is still concern because of their persistence in soil and water sediments and their capacity to accumulate in the trophic chain.

However there are many relatively new classes of pollutants, which we daily deal with, but are not still regulated by legislation. For these classes, fundamental research and appropriate monitoring plans are needed in order to understand the environmental distribution and fate, which are necessary to assess the effective risks for ecosystem and humans. CNR Institutes are carrying out research on many of these emerging environmental issues and, among them, two classes can be chosen as case studies, i.e. the engineered nanoparticles and the perfluorinated compounds. In the following sections a short critical review of the knowledge gaps will be presented in order to list future research needs for these two emerging classes of compounds.

4.1 Engineered nanoparticles

Anthropogenic nanoparticles (engineered nanoparticles; ENPs) are used in nanotechnology to create products used in various fields such as agriculture, electronics, biomedicine, manufacturing, pharmaceuticals cosmetics industry. The use of nanomaterials containing ENPs is expected to continuously increase in the near future. The nanometer size range (0-100 nm) means that nanomaterials exhibit properties and functions other than those owned by the same materials with larger diameter and these properties are

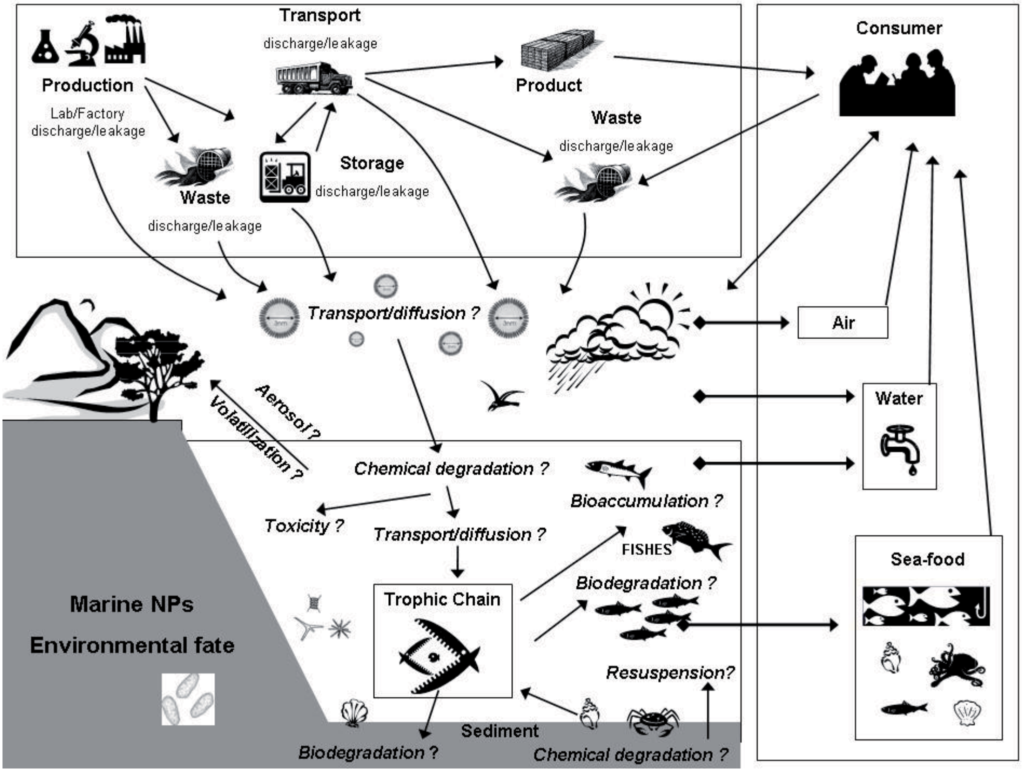

Figure 2: ENPs' environmental fate (figure adapted from National Institute for Resources and Environment, Japan http://www.nire.go.jp/eco_tec_e/hyouka_e.htm).

attributable to the increase of the surface area ratio and number per unit mass of ENPs, which lead to increased chemical reactivity, greater strength and electrical conductivity and, potentially, a more pronounced biological activity.

The extensive use of anthropogenic nanomaterials in large consumption products means that their transport, use and discharge is a potential new source of pollution in domestic sewage and industrial discharges, resulting in a diffused pollution of surface waters and transitional/coastal areas by ENPs. ENPs can be transferred to humans through food and several studies (103-120) demonstrate their potential risk for human health.

Today there are still many uncertainties about the environmental fate and toxicity of ENPs in aquatic environments because the methods for their determination and study in contaminated environments are not yet standardized.

The ENPs' environmental fate is extremely complex and the processes regulating ENPs' distribution in the different compartment are still not exhaustively investigated (Fig. 2). There are physico-chemical processes that can affect their potential environmental toxicity (solubility, aggregation, absorption, interaction with other toxic substances). The study of ENPs physico-chemical behavior influenced by abiotic factors in different matrices is fundamental to simulate scenarios in laboratory experiments.

According to Moore, some open issues can be suggested for future research projects(121):

- Which is the hydrodynamic behaviour of ENPs?
- Do they behave like larger natural particles?
- How do they associate with larger sediment

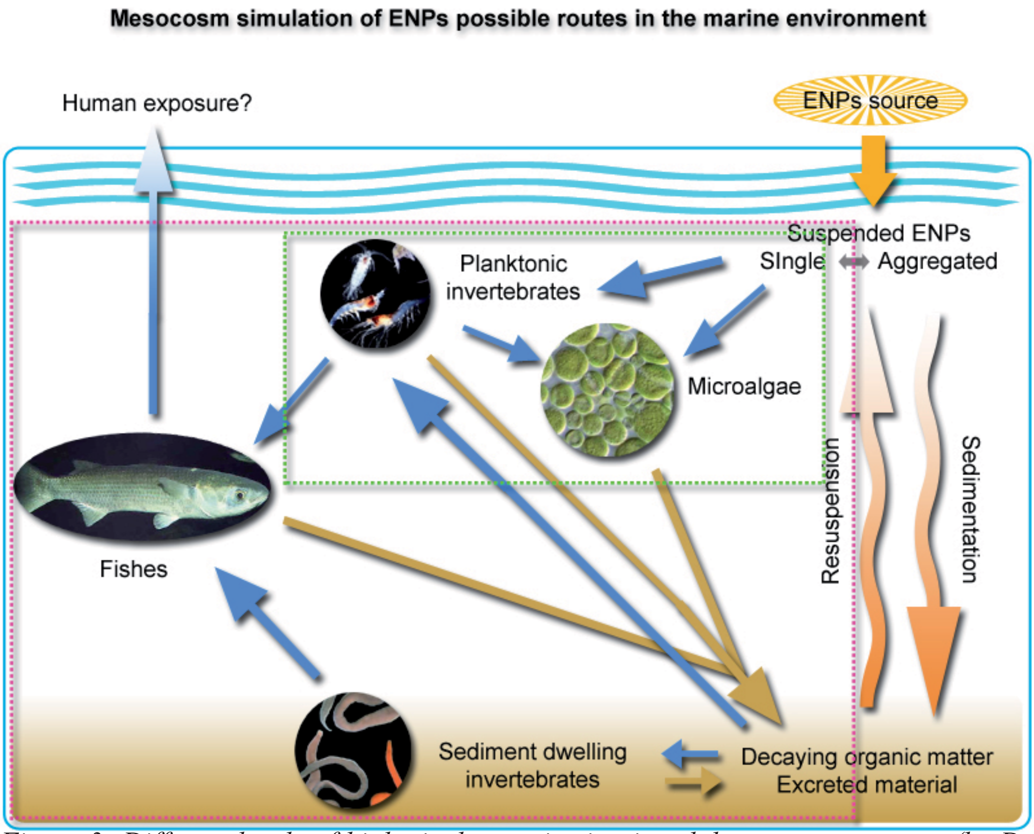

Figure 3: Different levels of biological organization in a laboratory mesocosm (by Dr. Giovanni Pavanello, ISMAR).

and natural colloidal particulates?

- Do they bind lipophilic organic and metal pollutants, and what are the routes of nanoparticle uptake into biota?
- Do ENPs-linked chemical pollutants show enhanced toxicity?
- Are the particle size and surface properties significant factors in determining toxicity in aquatic organisms?
- What is the implication of ENPs exposure for organisms health and ecosystem integrity?
- Can modelled fluxes and predicted impacts of ENPs help to provide an explanatory framework for their environmental behaviour and possible impacts?

All these gaps could be filled by planning an appropriate laboratory experimental model which should involve different expertises available in the CNR Institutes, with the cooperation of other national and international research institutions.

For the evaluation model, different classes of anthropogenic ENPs representative of potential future scenarios of use and impact in the aquatic environment should be chosen. Their interaction with various compartments of aquatic ecosystems (water and sediment), in varying chemical and physical conditions (pH, temperature, ion concentration, natural matter concentration) should be determined.

For each ENPs' selected class a standardized protocol for measurement must be developed for each different environmental matricx (water, sediment and biota), including all the possible chemical and physical interactions between ENPs and the different matrices. One approach could be a careful and critical analysis/testing of ENPs' "detection" methods already used in biomedical field (Environmental Scanning Electron Microscope-ESEM, Field-Emission Gun-Environmental Scanning Electron Microscope-FEG-ESEM, Scanning Transmission Electron Microscope-STEM, Transmission Electron Microscope-TEM, Scanning Electron Microscope-SEM, Atomic Force Microscope-AFM, Scanning Probe Microscope-SPM, Confocal Laser Scanning Microscope-CLSM and MultiPhoton Microscope-MPM) in order to adapt them to the specific peculiarities of the environmental matrices.

A crucial issue for a reliable risk assessment on ecosystems is the selection and standardization of ecotoxicological bioassays at different levels of biological organization (molecules, cells, organisms), which must be representative of different aquatic environments and ENPs categories selected as references standards. The selection of model species should be large enough to ensure a good representation of the different compartments of the most representative reference ecosystems.

Although it is reasonable and practical to predict the potential environmental risk on the basis of bioassays with single species models and/or battery of them, it is equally important to investigate how these supramolecular entities interact at ecosystem level. For this purpose, investigation protocols and exposure methods using realistic ENPs concentrations for various environments should be developed using laboratory micro and mesocosms containing sediment, microorganisms, algae, invertebrates and vertebrates, with different levels of ecosystem complexity. (Fig. 3).

The mesocosm study can also supply information on the accumulation and eventual biomagnification along the trophic chain. These data, together with human dietary and environmental exposure studies, are needed to get a reliable risk assessment for human health.

4.2 Perfluorinated compounds in the water environments

Perfluorinated alkyl substances (PFASs) are fluorinated man-made chemicals with unique molecular properties, chemical and thermal stability, water- and fat-repellent properties that make them and their derivatives useful in different industrial and household applications. They are widely employed for impregnation of textiles and paper, for cleaning aids, for fire fighting foams, for metal surface treatment and in the production of fluoropolymers.

Among PFASs, perfluorooctanoic (PFOA) and perfluorooctanesulfonic (PFOS) acids are two persistent and bioaccumulative end-stage metabolites of particular concern for human health and the environment due to their potential toxicological effects on animal and also human endocrine system; to date, there is no evidence of biodegradation of these chemicals.

Recent concerns with the toxic effects of PFOS and PFOA began in the early 2000s when the 3M Company, U.S.A, the major perfluorochemical manufacturer, decided to phase out the production of PFOS related products. However, there are still a number of industries, such as the semiconductor etc, that still use PFOS in their production (122) and manufacturers that use and produce PFOA and its related products for consumer usage. Evidence is accumulating regarding their persistence in the environment, potential for long-range transport, tendency to bioaccumulate, and potential toxicological effects (123-125). Due to their physico-chemical properties, these chemicals leaked into the water, they accumulate in surface waters, and water is the major reservoir of perfluorinated compounds (PFCs) in the environment, as well as the most important medium for their transport (126,127). Unlike other classical persistent organic pollutants (POPs), the anphiphilic PFCs have not been shown to accumulate preferentially in adipose tissue. They rather bind to blood proteins and accumulate in the liver of exposed organisms (128). Recent studies have indicated that PFOS can biomagnify in higher trophic organisms through the aquatic food chain (129,130).

PFOA is mainly used as adjuvant in the production of fluoropolymers such as polifluorotetraethylene and similar products used in clothing production, cosmetics and for non-stick cookware coatings. It was estimated that aqueous and gaseous emissions of PFOA or their salts originated in this production represent the majority (about 80%) of environmental release (126). It is also used (but in small amount) in industrial and consumer products (varnishes, inks, paper, floor polishes, cleaning formulation etc.) which represent some direct human exposure routes and diffusive environmental sources not normally considered. PFOA may also form as a degradation product of fluorotelomer alcohols found in a wide range of household and consumer products like hair shampoo, rug cleaners, and food paper products. These are volatile and can be carried for long distances by air currents (131).

In a European study on the major EU rivers carried out by the Perforce consortium (PERFORCE project), river Po watershed was identified as the dominant source of PFOA in Europe. River Po accounted for two thirds of the total PFOA discharge of the rivers studied (127). The PFOA concentration (200 ng/L) in the water of the Po river collected at the basin closure (Pontelagoscuro near Ferrara) was from 10 to 200 times higher than those measured in the other European rivers. This result suggested is the presence of an industrial source of PFOA in the Po watershed. Recent survey of perfluorocarboxylic and perfluorosulfonic acids concentrations in the Po watershed (33,132) confirmed the data of the PERFORCE project, and measured a PFOA concentration of 60-174 ng/l at Pontelagoscuro (FE). All tributaries, except river Tanaro, showed the PFOA levels typical of a diffuse pollution; on the contrary, the highest PFOA concentrations were determined in river Tanaro (1270 ng/L) and in river Po, downstream the confluence of the Tanaro river (60-337 ng/L), suggesting the presence of a point source for this compound that can be reasonably attributed to an industrial Teflon production site in the Alessandria county.

In the study carried out at the CNR-IRSA (33) an assessment of the contamination by perfluoroalkyl acids (PFAAs) in surface, urban and industrialized waste waters and drinking waters in the river Po basin was carried out. An HPLC method with ion trap mass spectrometry detection was developed for the analysis of perfluorinated carboxylates (from C_5 to C_{10}) and perfluorinated sulfonates (C_4 and C_8) with a LOQ of 2 ng/L. Water sample extraction was performed on weak anion exchange (WAX) cartridges.

In this work three kinds of waste waters, from textile industry discharge, urban wastewater treatment plant (WWTP) and mix urban-industrial WWTP, were analyzed because the industrial activity is considered the main source of PFOA and other homologues. PFOS was found at lower concentrations than PFOA, both in industrial and urban waste waters, This is probably due to the phase out of the

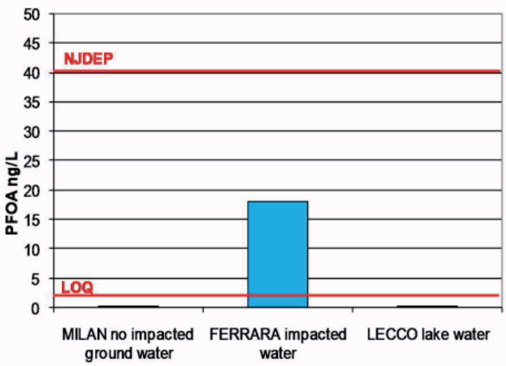

Figure 4. Perfluorinated concentration in tap waters. LOQ = Limit of quantification (2 ng/L); NJDEP = New Jersey Department of Environmental Protection guidance level (40 ng/L)

PFOS and PFOS-related compounds in the electrochemical production since 2002.

Three sources of tap waters (Lecco - produced from Lake of Lecco water; Milano - produced from ground water; Ferrara - partially produced from the Po river) were also analyzed. The only perfluorinated compound, detected in drinking water above the limit of quantification, was PFOA in the tap water of Ferrara which produces drinking water mixing ground and surface waters from river Po (Fig. 4).

To date, there are no international water guidelines for PFOA in drinking water but some single countries set up their own water guidelines. The strictest standard, (40 ng/L), issued by New Jersey in the USA, was not overcome by Ferrara tap waters. Nevertheless it is necessary to underline that this sampling campaign on drinking waters does not have any statistical representativeness, but it is only a baseline survey which needs further in depth enquiry.

Preliminary results highlighted the presence, in the Po river watershed, of point and diffused sources of perfluorinated compounds. PFAAs were measured in drinking waters produced from contaminated surface waters, revealing the risk for human consumption.

These preliminary campaigns, carried out in different periods and hydrological regimes, gave only a first look at the distribution of perfluorinated compounds in the Po watershed but no data have been collected for the rest of Northern Italy whose waters discharge in the High Adriatic Sea. The available data do not allow to estimate the risk of contamination for the aquatic species present in the transitional and coastal areas.

Furthermore the presence of intensive aquaculture activities in these areas could result in a source of exposure also for humans which, at the present state of knowledge, can not be envisaged.

Therefore, the overall goal of the IRSA research group will be to assess the environmental and health risks posed by the PFCs contamination in the river Po basin and in other river basins and coastal areas of the Northern Adriatic sea. In order to reach these objectives, the following detailed actions should be carried out:

- a comprehensive survey of the river Po basin , the coastal areas and lagoons of the High Adriatic Sea in order to identify critical areas and hot spots for PFC impact by:
  - Monitoring the river Po basin with downstream sampling of the main tributaries;
  - Evaluation of PFCs distribution in surface and groundwater around fluorochemical industrial sites in Northern Italy;
  - Monitoring of mollusc aquaculture areas in hot spot areas such as the Po Delta and Northern Adriatic Sea coast
  - Monitoring of raw and treated drinking water drawn from the Po river or groundwater impacted by industrial activities
- intensive monitoring of the critical areas in different hydrological regimes for the assessment of contamination and transport

processes;
- determination of PFCs levels in edible marine and lagoon bivalves, farmed or caught in the critical areas, in order to determine:
  - o PFCs accumulation in different sites, using two edible species with different geographical distribution (site comparison);
  - o PFCs accumulation in two different bivalves, addressing the possible existence of different patterns of exposure (clams in the sediment/mussels in the water column) and of bioaccumulation (species comparison);
- Estimation of PFCs risk exposure for humans as a result of the consumption of contaminated mollusks and drinking waters.

5. Future perspectives and developments

The term "emerging pollutants" means a continuously updated list of the most diverse molecules produced, used and eventually diffused in the environment. Such list includes synthetic surfactants, endocrine disruptors, pharmaceutical substances, perfluorinated compounds, industrial additives, new generation pesticides, etc, all substances which are not included in the lists of regulated priority pollutants and in ordinary monitoring plans. Considering also that the toxicity and the effects of certain new generation compounds are often not known or fully understood, it is evident how crucial it is to develop monitoring systems suitable to detect the possible responses to unknown toxic agents. In fact it is impossible to measure all the compounds present in a certain environmental compartment and we need to turn over our traditional approach in monitoring: first we measure the effects and then we look for the molecular agents which should be the cause of the effect itself. This revolution in the monitoring approach will be probably the most outstanding innovation in the next future.

At present, the procedures identified by the words "Toxicity Identification and Evaluation" (TIE) or "Effect Directed Analysis" (EDA) represent the state of the art in this field and for that reason we will present a short review on this topic in this last section.

The first identification process of "guilty" substances and its verification of the observed effects caused by these compounds dates back to 1983 (133). Subsequently taken over by the United States Environmental Protection Agency (US EPA), this process took the name of Toxicity Identification Evaluation (TIE). The classic TIE is divided into 3 phases. During the first phase (Fig. 5a), groups of active compounds are sequentially removed from the water sample with chemical treatment, as long as the toxic effect of the solution on the biological model system does not disappear. In the second phase substances are tentatively identified by the treatment that decreases the toxic response of the sample and the classes of chemicals identified are related to the biological responses. Finally the activity of each identified substance or substances mixture is confirmed using the same biological model.

The Effect Directed Analysis (EDA) procedure, which is the subject of EU research projects in the framework of WFD implementation, is the conceptual and technical evolution of the TIE approach. Like TIE, EDA is based on the use of the response of a biological reference system in combination with sample fractionation and the chemical analysis of individual fractions for identification of hazardous compounds in complex mixtures present in the environment. Figure 5b schematically

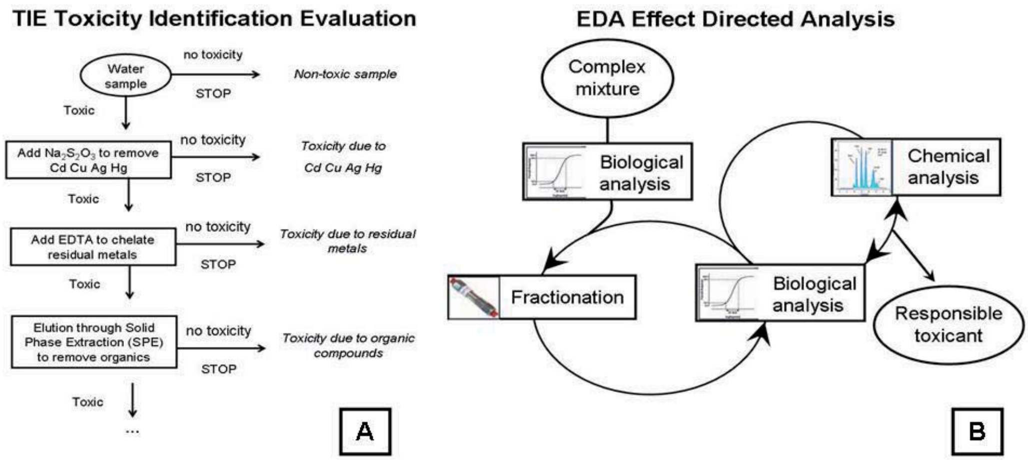

Figure 5: a-An example of TIE's first phase. b-scheme of EDA process.

describes the process which starts from the observation of a "macroscopic" effect on the biotic component in the investigation site. Through the use of a chromatographic separation systems, the mixture is divided into its individual components. Each fraction is tested for activity on in-vitro model systems, the nature of the compound is defined through analytical detection and recognition systems to determine the substance responsible for the effect.

Sample fractionation into individual classes of compounds is achieved using chromatographic columns with different stationary phases based on the principle that each compound has a specific affinity with the stationary phase column at a certain mobile phase composition. Therefore different types of serially connected chromatographic columns allow a sample basic components separation efficiency greater than that obtained using a single column, as in simpler TIE procedures (134,135).

The identification and confirmation of compounds in each fraction is achieved by using high-resolution mass spectrometry techniques, hyphenated with chromatographic systems; liquid chromatography (HPLC) is used for the separation and identification of polar compounds and metabolites which are more difficult to analyze but are much more biologically active than classic persistent organic pollutants.

In addition to a more rigorous procedure and a greater degree of complexity and precision in the sample fractionation compared to TIE, EDA introduces the use of in-vitro bioassays that allow a high number of spatial and temporal replicates and provide the capacity to run the tests in batteries, so getting a complete screening of the toxic activities of the sample fraction. As examples of in vitro tests used in EDA approach we can mention Yeast estrogen screen (YES), Yeast androgen screen (YAS), for the evaluation of endocrine disruption activity (136), bioluminescence tests such as CALUX, Microtox and Mutatox, assays on fish hepatocytes for physiological and accumulation studies: these are all sensitive, specific and rapid tests which allow to provide a full view on the biological activity of the compounds under investigation.

In parallel to those "classic" in-vitro tests, in recent years, the development of the "omics" disciplines helped to expand knowledge about the effects of many

toxic compounds: toxicogenomics and toxicoproteomics study the relationships between genome (or protein) structure and activity and the biological effects of exogenous agents. This means that if a compound shows, for example, a mutagenic effect, the magnitude of the effect can be determined by the gene expression analysis of an exposed individual.
As an example of the effectiveness of the integrated monitoring and analysis approach, we can mention the study of Keiter et al. (137) who adopted an integrated TRIAD and EDA method to identify the activity of so-called dioxin-like compounds (i.e. molecules whose steric and electronic structure is similar to those structural characteristics of dioxins which exert effects on biological systems) as the cause of a specific adverse effect on river organisms.
The EDA procedure is rather complex and time consuming and, of course, it is not suitable to meet the demand of fast and reliable techniques for early warning systems. Nevertheless, if integrated into routine monitoring programs as a complementary technique for investigative monitoring, it will be absolutely necessary in those situations where the toxic or other biological effect cannot be attributed to a known agent or source. Alternatively it could be used as a screening method to assess the risk of a particular pollution source that is crucial for an effective management of the territory with the aim of human health and environmental safeguarding.

With the contribution of WG2 participants: A. Barra Caracciolo (IRSA), E. Belluso (IGG) G. Caruso (IAMC), L. Da Ros (ISMAR), M. Faimali (ISMAR), M. Girasole (ISM), R. La Ferla (IAMC), M. Mancuso (IAMC), G. Mascolo (IRSA), V. Matranga (IBIM), L.S. Monticelli (IAMC), E. Morelli (IBF), P. Gualtieri (IBF), M. Pennisi (IGG), M. Polemio (IRPI), E. Rizzo (IMAA), E. Sacchi (IGG), R. Zaccone (IAMC).

With the contribution of: A. Baldi (Fondazione Italiana Endometriosi); E. Fommei (Pisa University and CNR-IFC), G. Iervasi, A. Pierini (CNR-IFC); S. Maffei, C. Vassalle (Fondazione "G. Monasterio" CNR-Regione Toscana, Pisa); C. Roscioli, S.Valsecchi, L. Viganò, D. Vignati (CNR-IRSA, Brugherio).

Keywords: water and soil monitoring, biomarker, bioassay, early warning system, emerging substances.

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