1 Environmental biological monitoring

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1 Introduction

The problem of environmental quality control is strictly related to the implementation of adequate experimental methods for the assessment of the actual state of health of ecosystems.

Ecotoxicology, as opposed to traditional toxicology, relates to a *series* of effects and responses to the contaminants of the ecosystem under study. Therefore, interpretation of the experimental data concerning the presence of contaminants in a given ecosystem requires data processing that is adequate to complex systems.

By *effect* we mean the kind of reaction shown by the ecosystem (not necessarily of a toxic nature); by *response* we mean the quantification (e.g. the percentage) of the kind of effect. The effects on the organisms may concern the reproductive system, motility, growth rate, etc.

Ecosystems, that consist of a biotic and an abiotic compartment, may respond in very different ways, and the onset of toxicity phenomena, as is known, may not occur until after some time.

However, we can assume that very low doses of contaminants do not generate negative effects on the organisms (biotic component). Every contaminant has a threshold, though, beyond which detoxification phenomena occur: these are just defense mechanisms that organisms may develop in the presence of these contaminants. Such mechanisms are often noticed in the different branches of the phylogenetic tree of the various species. In this respect, one of the most studied mechanisms is the one concerning metallothioneins (MTs) (proteins containing cysteine), which are able to link toxic metals [1].

The expression "actual state of health" we used in the first paragraph of this chapter aims at stressing the necessity of an evolution of scientific knowledge in the field of ecotoxicology and more importantly the necessity of employing alternative methods of environmental quality evaluation. These methods, such as biomonitoring, the use of biomarkers and biosensors in the environmental field, represent a sphere of important prospects of future developments.

WIT Transactions on State of the Art in Science and Engineering, Vol 30, © 2008 WIT Press
www.witpress.com, ISSN 1755-8336 (on-line)
doi:10.2495/978-1-84564-002-6/01

2 Traditional monitoring and biomonitoring

Traditional monitoring methods, as valuable and unsurpassed as they are in the sphere of Environmental Chemistry, do have some shortcomings:

1. considerably high costs
2. methodological problems
3. problems with the release of contaminants on an intermittent basis
4. effects on biological species
5. numerous and extensive samplings

Table 1 reports traditional analytical techniques for the analysis of environmental pollutants [2].

Biomonitoring has significant advantages over traditional analysis of abiotic matrices (water, sediments). Besides providing information on the bioavailability of contaminants, it simplifies the chemical analysis, eliminating the problem of the assessment of very low levels of contaminants; it prevents the risk of misinterpretations caused by sudden fluctuations in the environmental parameters at the time of sampling; thus, providing a measurement over time of the level of environmental contamination; it does not require numerous, extensive, and prolonged samplings in the areas under study. All the above goes to show the importance of biomonitoring as a means to control environmental quality.

The use of cosmopolite organisms to assess pollution has developed notably during the last few decades. Such organisms assume environmental contaminants and may be used as indicators of the bioavailability of a given contaminant over time, allowing – in certain cases – comparison between contamination levels in geographically different areas [2].

It is in this context the Organisation for Economic Co-operation and Development (OECD) countries have taken many initiatives for examining potentially dangerous products by proposing general programs for the monitoring and evaluation of environmental impact [2–7].

From an ecotoxicological perspective, we can consider as contaminants or producers of environmental stress, all chemical compounds that are fundamentally released into the environment as a result of human activities, and which cause damage to living organisms [2, 8].

3 European Union legislative framework on chemicals [9]

The chemicals that can be released into the environment are more than 100,000. There is a considerable lack of knowledge regarding most of them. It is in this context and framework that the enactment of an important document of the European Commission, the White Paper – Strategy for a future Chemicals Policy [9] is situated.

To cope with the obvious inadequacy of the knowledge relating to the environmental and toxicological properties of many of the chemicals present on the European market, the strategy proposed by the White Paper provides for the

Table 1: Analytical techniques for the analysis of environmental pollutants [2].

Pollutant	Reference instrumental methods
SO_2	Flame photometric (FPD) Gas chromatography (GC) Spectrophotometric (pararosaniline wet chemical) Electrochemical Conductivity Gas-phase spectrophotometric
O_3	Chemiluminescent Electrochemical Spectrophotometric (potassium iodide reaction, wet chemical) Gas-phase spectrophotometric
NO_2	Chemiluminescent Spectrophotometric (azo-dye reaction wet chemical) Electrochemical Gas-phase spectrophotometric Conductivity
Fluorides	Potentiometric method
PAH	High resolution gas chromatography associated with mass spectrometry (HRGC/MS)
PCDD	High resolution gas chromatography associated with mass spectrometry (HRGC/MS)
PCDF	High resolution gas chromatography associated with mass spectrometry (HRGC/MS)
Metals	Atomic spectrometry
Chlorine and hydrochloric acid	Volumetric method; spectrophotometric analytical method
Phosphorus	Gas chromatography X-ray spectroscopy

adoption of a single system for "existing" chemicals and for "new" chemicals, called REACH (Registration, Evaluation, Authorisation of Chemicals).

This system provides for the acquiring of factual data on the chemicals, following three levels of inquiry in relation to the type and quantity of the chemicals concerned, involving increased responsibilities both for industry and for public authorities.

The EU legislation in force [10] provides for a clear differentiation between "existing" substances, or those put onto the market before 18th September 1981

and "new" ones put on the market subsequently. The latter (around 2,900) have had to pass a health and environmental risk assessment, based on the results of experimental tests supplied by the manufacturing companies.

The system of notifying "new" substances involves the obligation on the part of manufacturing or importing companies to present to the relevant national authorities a number of detailed items of information about the physico-chemical, toxicological, and environmental properties of the substances – information which has been specified in detail in the various directives that have several times modified the EEC/67/548 directive [11].

For the other substances, which were already on the market before 18th September 1981 – the 100,106 substances defined as "existing" and listed in the European Inventory of Existing Commercial Chemical Substances – the available information is, by contrast, still scarce if not actually non-existent. They represent 99% of the volume of the substances marketed (these are 100,106 substances, comprising industrial chemicals, substances obtained from metals, minerals, and other products present in nature such as petroleum, substances derived from animals and plants, food additives, active substances of pesticides, fertilisers, medicines and cosmetics, natural monomers and polymers, and some waste products or by-products).

Of these 100 thousand substances, according to data supplied by the industry, around 2,500 chemicals with a high volume of production (over 1,000 tons/year), 15,000–20,000 chemicals with a "low" volume of production (between 10 and 1,000 tons/year) and almost 80,000 chemicals in quantities lower than 10 tons/year are manufactured or imported on the European market.

The chemicals manufactured or imported in the EU in quantities above one ton/year (including those with a high volume of production) amount overall to around 30,000 and for only about a hundred of these – those considered "priority" according to the regulation (EEC) no. 793/93 – is a risk assessment program provided, involving manufacturers, national authorities, and the European commission.

The White Paper presented by the European Commission starts therefore from the observation that some tens of thousands of chemical substances are manufactured or imported on the European market, for which we do not yet know the toxicological and environmental properties.

The slowness of the assessment program for the "priority substances," at present under way, was criticized both in the White Paper and in the report which the Commission presented at the end of 1998 [12]; from this document, it emerges that only 19 assessment reports were completed by the end of 1998 out of over 100 substances indicated as priority and that the time needed from when a chemical is inserted in a priority list to the completion of the report on the conclusions of the assessment varies on average from two to four years.

If this slowness is due in part to the complexity of the examination required, as well as to the meagerness of the resources devoted to assessment activities, it is also true that the subdivision of responsibilities between manufacturing companies and national authorities does not facilitate the speeding-up of the program.

While in the case of "new" chemicals, industry has everything to gain from cooperating with national authorities, supplying all the necessary data so that the products may be assessed and marketed as quickly as possible, in the case of "existing"

chemicals that are already on sale, the onus of proof falls *de facto* on the public authorities with no specific obligations for companies about the data to be supplied in order to allow an appropriate risk assessment. Proving an "unacceptable" risk for human health and for the environment therefore rests entirely with the public authorities.

The EU is in the process of making the most fundamental changes to its legislation on the management of chemicals for over 30 years. Final political agreement was reached at the Environment Council on 18th December 2006 after a severe period of negotiations between the key stakeholders (the European Union, Member States, the European Parliament, and in particular the Chair of its Environment Committee, Directorates-General, Environment and Enterprise of the European Commission, and others (i.e. industry, non-governmental organizations).

The final REACH text (more than 800 pages) was published in the Official Journal of the European Union on 30 December 2006 and can be found at: http://europa.eu.int/eur-lex/lex/JOHtml.do?uri=OJ:L:2006:396:SOM:EN:HTML REACH entered into force on 1 June 2007; it now replaces over 40 existing Directives and Regulations.

Another important issue is the one concerning the discussion of the problems inherent in assessing the environmental risk of persistent organic pollutants (POPs), which was the subject of a recent international convention in the United Nations Environment Programme (UNEP) (Stockholm Convention on POPs, 2001) [13].

3.1 Categories of contaminants

The compounds released into the environment as a consequence of human activities can be classified in two main categories: biodegradable substances and conservative substances [14].

Biodegradable substances represent the larger volume of human-generated wastes (domestic wastes, industrial wastes, etc.) and, depending on their origin, they are disposed of in the air, the soil, or water. They consist of organic material (rich in C, N, and P) liable to bacterial degradation through oxidative processes that reduce these organic compounds to soluble inorganic compounds (CO_2, H_2O, and H_3N). If the release of these compounds is very high, anaerobic activity occurs, with ensuing origination of degradation products (H_2S, HN, H_4C) that not only give off unpleasant smells, but are also toxic for many organisms.

Generally, uncontrolled release of biodegradable waste products, especially into water bodies, causes eutrophication phenomena and subsequently a decrease in the quantity of oxygen present in the medium in question; it can also bring about the production of toxic degradation compounds.

Conservative substances are not decomposed by bacteria or other short-term processes. These substances are typically very reactive toward plants and animals, sometimes causing considerable damages. There are three main categories:

1. heavy metals (Pb, Cd, Hg, Cr, Cu, Zn …);
2. halogenated hydrocarbons (HHC), dichlorodiphenyltrichloroethane (DDT), and polychlorinated biphenyls (PCBs);
3. radioactive compounds.

The components of these categories can be regulated by animals and plants to highly variable degrees, depending on the species, but always in an interval common to all species.

Halogenated compounds and metals are not eliminated; they accumulate over time in the tissues of the organism concerned and stay there permanently.

For instance, in aquatic systems, those organisms that are predators of bioaccumulators may have a diet rich in these conservative substances, which sometimes causes very high concentration levels of these compounds. Hence, there arises an exposure risk for trophically superior organisms (among which man) with subsequent problems concerning the release of these substances into the food chain [15].

4 Kinds of biomonitoring [2]

In general, *bioindicators* are organisms that can be used mostly for the identification and qualitative determination of human-generated environmental factors [15–17], while *biomonitors* are organisms mostly used for the quantitative determination of contaminants and can be classified as being *sensitive* or *accumulative*.

Sensitive biomonitors may be of the *optical* type and are used as integrators of the stress caused by contaminants, and as preventive alarm systems. They are based upon such optical effects as morphological changes in abundance behavior related to the environment or upon such chemical and physical aspects as alteration in the activity of different enzyme systems as well as in photosynthetic or respiratory activities.

Accumulative bioindicators have the ability to store contaminants in their tissues and are used for the integrated measurement of the concentration of such contaminants in the environment. Bioaccumulation is the result of the equilibrium process of biota compound intake/discharge from and into the surrounding environment. Bioaccumulation is the enrichment of a substance in the organisms through every pathway (respiration, food, dermal exposure).

The first studies of bioindicators date back to the 1960s. Beginning with the theoretical calculations of Stöcker [18] and Phillips [19, 20], we can define the main characteristics of a bioaccumulator.

Bioaccumulators must [2]:

- accumulate the pollutant without, however, being killed by the levels with which it comes into contact;
- have a wide geographical distribution;
- be abundant, sedentary, or of scarce mobility, as well as being representative of the collection area;
- be available all year round and allow for the collection of sufficient tissues for analysis;
- be easy to collect and resistant to laboratory conditions, as well as being usable in laboratory studies of contaminant absorption, if necessary;

- have a high *concentration factor* (CF) for the contaminant under study, and thus allow direct analysis with no prior increase in concentration;
- have a simple correlation between the quantity of contaminant contained in the organism and the average contaminant concentration in the surrounding environment;
- have the same contaminant content level correlation with the surrounding environment in every site studied and under any condition. This must be true for all organisms examined.

5 Reference levels, concentration factor, and biomagnification

An ecosystem must be understood as an entity that is relatively stable over time and having functional autonomy where there is an energy flow between organisms belonging to a complex trophic network.

The interactions that may occur among organisms are numerous and therefore also complex ones. When performing biomonitoring and traditional monitoring programs it is very important to consider these factors. Slobodkin [21] distinguished three different kinds of interaction among organisms:

1. Alterations of an indirect type that is when an organism alters the physical environment of another organism. For instance, the trees in a wood cast shadows and therefore reduce the photosynthetic activity of the surrounding plants;
2. Alterations (of an indirect type) of the physico-chemical environment, such as the increase in oxygen concentration, as a result of photosynthesis, in lake ecosystems on the part of unicellular algae. This oxygen is available for the respiratory activity of other organisms;
3. Exchange of chemical compounds, elements, or energy among organisms; many wild animals, for instance, constitute the food of both big and small predators, while the seeds of many plants provide several species of birds and mammals with energy, proteins, and vitamins (alterations of a direct type).

So, biomonitoring can be considered alongside traditional monitoring as it allows environmental quality assessment over wide geographical areas and an integrated measurement *over time* of the presence of contaminants.

For a variety of reasons, it is of fundamental importance to define the reference levels for pollutants in an ecosystem when making biological monitoring studies to:

1. evaluate the state of conservation or degradation;
2. predict the incidence of possible future human activities in order to establish the necessary interventions;
3. control evolution over time, using monitoring programs, if necessary.

To correctly evaluate the degree of contamination in an ecosystem, or to carry out biomonitoring operations, it is necessary to first establish the *background level* (BL) of the contaminant, both in the environment (air, water, soil), and in the organisms [15]. The BL may be interpreted in different ways: it may be understood as a *pre-industrial level* (prior to any human activity); as a *natural level* (the average conditions of an area or a region where there may be human activity, but which is in a good state of conservation); a *standard level* (based upon global geographical references); or even a *zero level* (the concentration of an element in the environment or in an organism prior to the development of a particular activity that is independent of the degree of conservation) [15].

Once the BL has been established, we use the CF to evaluate the state of conservation of an ecosystem, or to monitor its state. It is also called "contamination factor" mainly when this refers to biota/biota or abiotic compartment/abiotic compartment ratios of contaminants concentrations.

The CF is the relationship between the level of a contaminant found in the biota or environment and a reference value that represents a determined stage (pre-industrial, natural, zero):

$$CF_b = \frac{C_b}{BL_b} \quad \text{or} \quad CF_a = \frac{C_a}{BL_a}$$

CF = the contamination factor for the biota ($_b$) or the environment (air, water, soil) ($_a$); C = the concentration of contaminant in the biota ($_b$) or in the environment ($_a$), respectively; BL = the background level, of the pollutant in the biota ($_b$) or in the environment ($_a$), respectively.

If the BL is a reference of the zero level, it will allow us to observe the evolution of a pollutant (in terms of both space and time), during a contamination process.

In this case CF can be used as:

$$CF = \frac{Cb_{2A}}{Cb_{1A}}$$

Cb_{1A} = the concentration of pollutant present in the biota at the time 1 in the site A; Cb_{2A} = the concentration of pollutant present in the biota at the time 2 in the site A.

This concept may also be used to observe the decontamination rate in an ecosystem (positive impact). This signifies that the CF can be used also when, during comparisons between different environmental situations, no data is available for the BLs.

The CFs, in the monitoring programs, are also intended as the ratio between the concentration of the pollutant in the biota/BL of the contaminant in the environment at the same time of sampling:

$$CF_b = \frac{C_b}{BL_a}$$

For example, the Pb concentration measured in a mollusc tissue vs. the Pb concentration in the seawater soluble fraction. This reflects the concentration capabilities of the biomonitor with respect to the surrounding environment. It is generally correlated with the bioavailable fraction of the contaminant in the environment.

The system for environmental classification is realised by starting with the CFs obtained for each contaminant present in the environment or organisms. When evaluating the CFs obtained, it is also necessary to take into account the uncertainties that derive from the following: sampling; space, and time variations for the samples; the age and condition of the organisms, etc. In general, a CF that is above a given number (generally 1.5, 2, or 3 times the BL), is taken to be the minimum level under which it is no longer possible to refer to certain contamination. The qualification of a contamination situation may follow a linear scale, or, in high-level pollution conditions, a scale of the exponential type. Of course, the CF of each contaminant varies according to the species.

It is generally assumed that the release of the compound during the time unit is proportional to its concentration in the tissues. In these conditions, the higher the absorption constant of the contaminant and the lower the release constant, the higher the CF will be: the CF is the result of the processes of intake/discharge of the contaminant on the organism tissues. When using the CF as an indicator for the assessment of the quality of an ecosystem, we must take into account the possibility of self-regulation phenomena (detoxification) on the organism tissues which might alter the CF values.

The accumulation efficiency of an organism can be measured performing accumulation kinetics experiments, by keeping the contaminant concentration in water or air steady at levels that are not harmful to the organism itself, so as to determine the accumulation (or the mass growth) of the organism in terms of time (saturation curve). A contaminant can enter an organism through respiration (reversible process) or through food. Fig. 1 shows a general outline of the bioaccumulation phenomena.

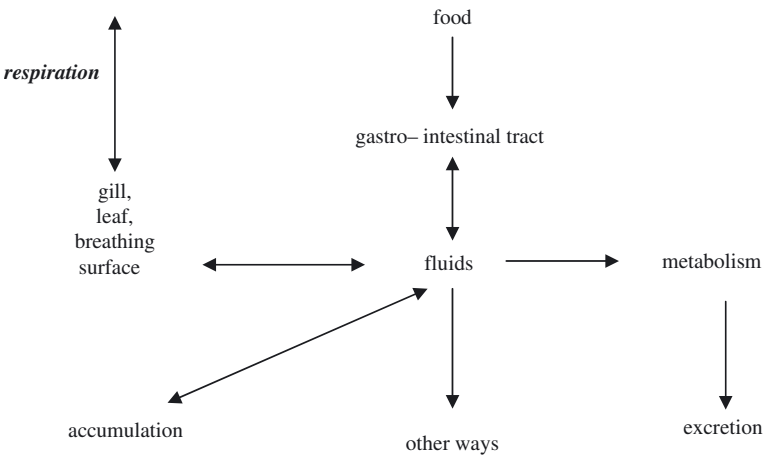

Figure 1: The outline of bioaccumulation phenomenon.

Bioaccumulation is thus understood as the intake of a contaminant through all possible pathways. *Bioconcentration*, on the other hand, is the direct intake of a contaminant exclusively through respiration.

The concentration of a number of compounds (as for instance mercury) accumulates every time it passes through the food chain. This phenomenon is known as *biomagnification* or concentrations along food chains.

However, biomagnification is a relatively rare phenomenon. One should demonstrate that the food pathway for the intake of a contaminant is clearly predominant over the others (especially respiration). Moreover, the accumulation of contaminant should increase with the passage from prey to predator, which is not always the case.

Some contaminants have much higher transfer efficiency than others: in these instances the losses through the passage from a compartment to another along the trophic chain are negligible. Such is the case of dichlorodiphenyldichloroethane (DDD), an insecticide of the DDT family, that was studied by Hunt and Bischoff in 1960 [22]. It was observed that the concentration of DDD increased as a consequence of the passage from water to aquatic organisms and from prey to predator. The concentration increased even more in the passage from fish to fish-eating birds (*Aechmophorus occidentalis*) situated at the top of the trophic chain.

In particular, the authors found that the DDD levels in the water of the Clear Lake, California were 0.02 ppm; in plankton 5.3 ppm ca.; in small fish 10 ppm; in predatory fish 1,500 ppm and in the western grebe fat 1,600 ppm. All this highlighted the biomagnification phenomenon.

The biomagnification of hydrophobic substances with a high resistance to degradation, such as DDT or PCBs, can be ascribed to the different physiology of the various organisms. If we consider that the air/water partition coefficients (K_{AW}) (at 20 °C) of DDT and PCBs are 10^{-2}–10^{-3} we can infer that their water concentration is 100–1,000 times higher than air concentration. Therefore, the transfer capacity of the contaminant from and to water organisms is two–three orders of magnitude higher than it is in air. As a matter of fact, aquatic organisms provided with gill have to extract the oxygen they need from water, when the oxygen concentration in water is 1/27 compared to air concentration (in equilibrium conditions at 20 °C). The gill must therefore work incessantly in order to extract a sufficient amount of dissolved oxygen from the water, which entails also a high exchange efficiency of the contaminants. For fish, the deposit compartment of contaminants is non-polar lipids that are easily assimilated by predators. This increases the possibility of biomagnification phenomena.

Therefore, assimilability is the decisive factor for biomagnification. We can observe that the levels of hexachlorobenzene (HCB), DDT, and PCBs in the grass of a meadow have values that can be compared to those present in fish, although the concentration of these contaminants are 100–1,000 times lower than those that can be found in the aquatic compartments. The above-mentioned contaminants accumulate in wax and possibly also in the cutin present in grass. These elements are not easily assimilated by ruminants, and therefore there is no biomagnification, due to the low transfer efficiency of the contaminant from the grass to

the ruminant. The accumulation of contaminants in the waxes of terrestrial plants reduces sensibly biomagnification in herbivores [23].

Being lipophilic, contaminants often accumulate in the fat tissues of the organisms. The CF of an organism grows in proportion to the growth of K_{OW}, which is defined as the water/*n*-octanol partition coefficient. This partition coefficient is used because it is highly assimilable to lipids; it has a long hydrocarbon chain and has an alcoholic terminal function.

The measurement of the K_{OW} is considered to be very important, since several American, European, and Japanese environmental bureaus require them as indicators for the assessment of the quality of new compounds that are being put onto the market.

However, some recent works stress the fact that there has been a high level of uncertainty in reported octanol–water partition coefficients and aqueous solubilities (S_W) for DDT and dichlorodiphenyldichloroethylene (DDE) over the last six decades [24]. There was found four orders of magnitude variation in the K_{OW} and S_w database values for these hydrophobic organic compounds. The whole data quality seems to need to be reconsidered [24].

Biomagnification, however infrequent, can occur with methylmercury, the organic form of mercury found in water ecosystems, where it is very stable. Methylmercury is highly photolabile and is therefore found in places with scarce luminosity.

We all remember the tragic accident at Minamata Bay (Japan), where a chemical industry employing Hg^{2+} as a catalyst in the production process of polyvinyl chloride used to discharge wastes contaminated with mercury into the sea. Methylmercury bioaccumulated in fish, and fish was the staple food for the people living along the coast. The concentration of mercury in the fish was higher than 100 ppm (in the USA the recommended limit for mercury in fish for human food use is 0.5 ppm).

During 1950s, thousands of people living in Minamata suffered from mercury poisoning and hundreds of them died.

Another more recent disastrous accident is the one involving the petrochemical industry Enichem of Priolo, (Sicily): up to 2001, mercury used to be illegally disposed of in the sea. The levels of mercury measured in the sea were 20,000 times higher than the ones allowed. Congenital deformities in fetuses had been reported for some time in the area.

The K_{OW} of methylmercury is ca. 1, which means that its hydrophobicity is very low and its bioaccumulation capacity is virtually negligible. However, it penetrates cellular membranes easily (since it does not differentiate lipids from water, as indicated by its $K_{OW} \approx 1$) thus forming stable complexes with thiolic groups of the proteins inside the cells where it builds up.

Transfer efficiency from a trophic level to the next can be measured by the trophic transfer coefficient (TTC), which is the ratio between the concentration of the contaminant in the tissues of the consumer and the concentration in the tissues of the food (prey) [25]. Therefore, if the TTC value is lower or equal to one, there occurs no biomagnification phenomenon. If it is higher than one,

there is biomagnification. Suedel *et al.* [25] detected TTC values as high as 100 for methylmercury (full-blown biomagnification). For several organic compounds studied (atrazine, chloro-dioxines, polycyclic aromatic hydrocarbons (PAHs)) the tests for biomagnification had negative results (TTC $\leqslant$ 1). Other contaminants (DDT, DDE, and PCBs) show TTC values ranging between 0.1 and 10. PCBs (highly hydrophobic) have TTC values as high as 10 in the passage from small to big fish, and it reaches an enrichment factor of 100 in the passage to dolphins and fish-eating birds [26, 27].

Another example of accumulation in the trophic chain is the one concerning radioactive depositions over the Mediterranean area after April 1986 (Chernobyl accident). The rapid removal of Cs-137 from water by biological activity has transferred this radionuclide to the pelagic and benthic communities [28]. Transfer of Cs-137 along the marine trophic chain in the Po delta (Adriatic sea) was found in 1990. Levels of Cs-137 (Bq/kg wet weight) were: 0.07 for plankton; 0.5 for *Merluccius merluccius*, and 0.6 for *Sardina pilchardus* [29].

6 Sampling problems

Before the assessment of the BL of contamination of an ecosystem, it is necessary to clearly define the area under study and the area that can be affected by a possible source of impact.

The area of influence depends on several factors:

1. the magnitude of the impact (e.g. volume of the pollutants released in the atmosphere);
2. the characteristics of the receiving body (e.g. wind direction);
3. the time scale that will be used.

The area of influence of a group of contaminants varies depending with time. Another problem is the representativeness of the samples and the intensity of the sampling. When establishing the number of samples, it is necessary to consider the size of the area of influence and the actual presence of the species under study in the area concerned.

The necessary number of samples changes with each kind of material that is analyzed (atmosphere, sediments, biota) and with each contaminant studied, since they have different affinities both regarding their bioconcentration capacity and their distribution in the ecosystem. As a general rule, the less we know about a contaminant, the more samples we need.

Bros and Cowell [30] developed a technique that employs the standard error (SE) of the mean to resolve statistical power in order to determine the number of samples needed based on number of species, number of individuals, biomass, diversity, and evenness. This method uses a Monte Carlo simulation procedure to generate a range of sample sizes vs. power. As the number of samples is increased, the rate of increase of SE declines, and eventually becomes near to zero level; the

point where this occurs corresponds to the minimum number of samples required. Generally, the less abundant the pollutant, the greater the number of samples required to assess its level.

When quantifying the BL of a contaminant in a given organism, we refer to the mean concentration of the contaminant in the tissues of the organism. It is important to take into account the condition index (age, sex and maturity, size, etc.) of the population under study. These elements can alter significantly the concentration of the pollutant in the organism. For example, in molluscs the concentration of the contaminant depends on the weight (size). Therefore, in biomonitoring studies using molluscs, the concentration/weight factor is determinant and important for the processing of data (see further on).

The BL can also refer to the natural concentration of the pollutant in a given organ (brain, liver, pancreas, thallus, etc.) or to an analytical fraction that corresponds to a given extraction process (intra-extracellular, lipid fraction, etc.). This is because those organs, beside having a particular bioaccumulation ability, present less difficulties when chemical analyses are performed; or also for the different biological meaning that this localized bioaccumulation takes on. The data obtained this way are generally more stable than those obtained from the analysis of the whole organism.

6.1 Sample collection

The methods and materials used to collect, store, and transport samples should be considered carefully. Sampling devices and their materials of construction should have to be evaluated under specified conditions. Contamination by sampling devices and materials can contribute relatively large errors in comparison to analytical procedure, especially when the analytes of interest are at low concentrations. Sampling protocol should be based on labile analytes to be measured. The term labile is hence regarded as the metal forms with the highest probability that the analyte concentration change prior to analysis.

Composite sampling defined as combining portions of multiple samples is of advantage in a monitoring program. Even when each individual test sample continues to be the material of study used in many biomonitoring studies composite sampling frequently replaces collection of individual specimens [31].

Composite samples are often used to reduce the cost of analyzing a large number of samples and also to diminish intersample variance due to heterogeneity of the sampled material. Statistical evaluation of the results obtained in composite samples indicate less mean square error in the frequency of analyte occurrence than in the approach of sampling and analyzing one individual from each one of the multiple populations collected.

Another advantage of composite sampling is that it may also increase the amount of sample available for analysis, especially when each individual furnishes too little quantities of material of study.

After sample collection, appropriate conditioning and storage precautions are of concern in order to minimize the risks of analyte loss or contamination.

For speciation studies of biological materials more stringent conditions have to be fulfilled during sample collection, pretreatment, and storage. It is of relevance to maintain the integrity of the chemical species during and after sampling. Changes in parameters such as temperature, ionic strength, pH, redox potential, oxygen level, irradiation with UV light, etc., to which the samples are exposed, can influence the distribution of chemical species.

The choice of sample storage containers is also a critical factor, being deep freezing samples an appropriate technique to perform immediately after collection to minimize bacterial or enzyme degradation.

6.2 Sample preparation

Until the middle of the last decade sample preparation procedures for biological materials fell into two categories. Firstly, analytical methods with minimum sample treatment (nearly intact sample) and secondly, those methods where an important sample treatment such as separation, extraction, or destruction of the organic matter is necessary before determinations can be carried out.

Sample preparation in inorganic analytical chemistry is generally concerned with digestions, which principally serve to liberate target compounds from the sample matrix and convert the various chemical forms of the analyte to a uniform species [31].

Analysis of plant materials and animal tissues usually involves destruction of organic matter. Wet acid and dry ashing techniques are commonly used for this purpose but the former has been gaining more acceptance during the last years. Digestion by refluxing in boiling concentrated acids such as nitric acid or nitric/perchloric acids is the conventional technique used in biomonitoring studies but special microwave heating for acid digestions has lastly increased the speed and efficiency of the operation (for more details on digestion procedures see Chapter 5).

Sample preparation in organic analytical chemistry mainly comprises extractions, which serve to isolate components of interest from a sample matrix because most analytical instrumentation cannot handle the matrix. Extraction techniques using large amounts of organic solvents in analyte laboratories such as the Soxhlet extraction method are still used. However, several disadvantages of solvent extraction in routine analysis are known: sample preparation is time consuming, employ multi-step procedures with high risk of analyte loss and are rather expensive [31].

In biomonitoring the main drawback of the entire process would probably be that most of the analysis time is consumed by sampling and sample preparation, mainly because of the large number of samples to handle and process. Microwave assisted extraction (MAE) techniques are an interesting alternative for separation of organometallic compounds in environmental and biological samples. Extraction in an open microwave oven is of great advantage for dissolving samples because it offers reducing extraction time and reagents, thus reducing contamination problems. Furthermore, procedures for sequential extraction using microwave heating under controlled conditions were also established [31].

In the last years a novel technique, solid phase microextraction (SPME), regarded as a sorbent extraction technique and a solvent-free sample preparation, has been applied for trace element speciation in environmental and biological samples. One of the main advantages of SPME in metal speciation is that it is a non-exhaustive technique permitting equilibrium of the target compound between the free and bound-to-matrix forms be practically undisturbed during the extraction procedure [31].

Volatile and non-volatile organometallic compounds can be collected by fibers placed into a tip of the SPME system at the entry of gas chromatography (GC) and liquid chromatography (LC) instruments, respectively, used for their separation. Only a soft digestion of the sample to drive the analytes into the liquid phase is needed for immersion of the tip, though in other cases the fiber is in contact with the gas phase of the headspace in the chamber containing the sample solution [31].

6.3 The use of certified reference materials

National and international marine monitoring programs have been initiated worldwide to assess the quality of the marine environment. In environmental analysis *Reference Materials* (RMs) play an important role to achieve quality in the results; therefore, an appropriate use of reference standards must be emphasized.

Definitions of some useful terms related to RMs are given in ISO Guides [32–34]. RM is a material or substance one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of an apparatus, the assessment of a measurement method, or for assigning values to materials whereas a *Certified Reference Material* (CRM) is a Reference Material, accompanied by a certificate, one or more of whose property values are certified by a procedure which establishes its traceability to an accurate realization of the unit in which the property values are expressed, and for which each certified value is accompanied by an uncertainty at a stated level of confidence.

Traceability implies that a measurement result should be related to stated references, and therefore, the value reported in the certificate of a CRM is traceable. So, when CRM are used in a method the user has to demonstrate its traceability. *Traceability* is defined as a property of the result of a measurement or the value of a standard whereby it can be related, with a stated uncertainty, to stated references, usually national or international standards, through an unbroken chain of comparisons.

Certification of a Reference Material is a procedure that establishes the value(s) of one or more properties of a material or substance by a process ensuring traceability to an accurate realization of the units in which the property values are expressed, and that leads to the issuance of a certificate.

The large array of matrices encountered in the environment makes necessary the use of a very wide variety of RMs derived from different sources, related to the intended purpose of the user. Advantages and choices of CRM as well as the procedure for carrying out the measurement are described in the International Organization for Standardization (ISO) Guides [32–34].

CRM chosen must adequately match the material to be analyzed, and it also implies that the levels of the analytes or target compounds have to be similar to those of the real samples used for analysis. Traceability of CRM will be questionable when the matrix and analyte contents are too different from those of the analyzed sample.

The contents of trace, minor, and major elements in CRM for the quality control of marine and estuarine monitoring programs include biological, sediment, and seawater samples. Technical information and purchasing conditions are provided from the main suppliers by request.

Usually, most elements exert their biological and environmental impact as components of macromolecules, or linked to small organic compounds, or according to a specific oxidation state. Therefore, not only total element determinations are of interest any longer but also identification and quantification of element species (speciation).

In the last twenty years, speciation analysis in the environmental and life sciences has mainly focused on the analysis of relatively stable organometallic compounds such as some organic forms of mercury, tin, or arsenic, and oxidation states: Cr (III)/Cr(VI).

Today, there is a great interest in speciation studies of many other elements present in the marine environment and biota. It is also necessary that availability, and distribution, transport, transformation, and fate of chemical species of elements regarded as toxic, rather than of the total amount of the elements, be studied in very complex systems and samples taken from biotic and abiotic sources. Unfortunately, there is a limited number of available CRM to fit these purposes. Existing CRM are intended to be used for trace element speciation and fractionation purposes. Explanation of these two fundamental concepts has been extensively treated in a publication of the International Union of Pure and Applied Chemistry (IUPAC) [35].

7 Determination of background levels (BLs)

The determination of BLs [15] is fundamental to determine the degree of enrichment of the contaminants in the environment or in the biota. Generally, the data used as BLs come from two different sources:

1. mean values used as general reference levels
2. values determined in their area of study

Both procedures are valid and the choice between them depends on the goal one is trying to achieve. Using general reference levels local variations will be ignored. On the other hand, using the pre-industrial levels of the areas concerned, local variations will actually be emphasized.

However, for an exact environmental assessment and/or monitoring of a given geographical area, it is generally preferred to use the values determined in the

specific area of study, even though this entails higher costs, sampling and laboratory work. In this approach, it is important to minimize the effect of the variability of the samples in the selected sites.

The difficulty with these methods of approach to study lies in the ability to distinguish between the variability due to contamination and natural variability, as well as to report data on a regional scale. The key for a correct definition of BL is to select unpolluted sites. This selection can be performed by choosing:

1. for each contaminant the sites that are not polluted by this particular contaminant;
2. the sites those are also free from contamination of typically polluting elements.

When selecting uncontaminated stations, before sampling a geographical area, it is necessary to follow a number of preliminary procedures [15].

In accordance with the information available, a series of stations (or sites) can be selected that are considered representative of natural levels. This decision entails a high degree of risk if there are no exhaustive preliminary data on the area of study.

The selection of the sites can be made using multifactorial techniques. With this method, clean stations are selected after being grouped through multifactorial analysis (principal component analysis (PCA), dendrograms, etc.) applied to a matrix of data that are linked to the quality of each ecological station.

Elements of segregation can be used, together or separately, such as the physico-chemical parameters of the environment, the concentration of the pollutants in the environment and organisms, the frequency of the species of bioindicators, etc.

However, this method presents some problems. The results can be difficult to interpret and may have low grouping levels, especially if the majority of the samples have low levels of contamination. Furthermore, contamination is distributed along different gradients that depend on the natural variability of the physico-chemical conditions of the environment (air, soil, water). This method is more effective when the areas of study present more homogeneous environmental conditions and the contamination points are more punctual and intense.

Another method for the selection of clean sites involves bioindicators. Ecological stations are selected on the basis of a possible manifestation of stress in one or several species of environmental quality indicators.

The stress to be measured may be biochemical, physiological, or structural (e.g. chlorophyll degradation). Usually, several bioindicators are used belonging to different biological species present in that environmental compartment and responding to pollutants in a complementary way. This method allows the evaluation of the degree of natural preservation of the selected sites with a reasonable degree of approximation.

When the contaminant type is known, some indicators can be used more effectively and this allows the use of less costly methods. If the contaminants are not known and the information on the tolerance level of the species is scarce, a multiple survey and possibly toxicity tests are necessary.

A multiple level survey entails the study of possible indicators and changes in the behavior of the organisms. The indicators have to show biochemical, genetic, morphological, or physiological changes (biomarkers). The behavior indexes are determined by the changes of given species, dynamic populations, or communities (e.g. freshwater macroinvertebrates).

The biomonitoring of communities provides information on the magnitude and the ecological effects of the contaminants in an ecosystem. The cause/effect relationships are difficult to establish and the knowledge in this respect is still dubious, since there are many factors affecting the community-contaminants system. Therefore, the use of bioindicators at different organizational levels (for instance: individuals, species, communities, ecosystems) is more advisable.

If the choice of the uncontaminated stations concerns a given contaminant, there are different statistical methods that can be used, such as the use of data populations with a coefficient of variation (CV) of approximately 60%. This method is used for the assessment of the natural geochemical BL in soils and consists on the progressive elimination of the higher values of the contaminant under study until a CV of 60% is reached. Such population distribution is considered normal [14, 15].

Many ecotoxicological studies use the determination of homogeneous populations within a number of data belonging to a given site, employing graphs and diagrams. These studies rely on the analysis of distribution curves of cumulative frequencies transformed in a log-normal distribution (cumulative frequency curves). It is thus possible to distinguish homogeneous populations (straight lines in the diagram) that correspond with the different contamination levels: base level, middle level, and contaminated. The problem with this method is that it is necessary to have a significant amount of data in order to have a valid graphical interpretation of the populations.

Another method involves normal populations. In order to solve the problem of size in the number of data – which is a common problem in most works, modal analysis methods are also applied. Such methods are often used in demography studies employing adequate software. For instance, NORMSEP (SEParation of the NORMally distributed components of size-frequency samples) is a program that transforms the frequency of the concentrations obtained into a distribution of normal components, thus allowing a differentiation between the various homogeneous populations that may coexist with the complete series of data. Thanks to this method it is possible to work even with a reduced number of samples.

The use of regression techniques is applied between a stable element that is not affected by anthropic activity and the rest of the contaminants. In this instance, the clean stations will be those presenting a confidence level of 95% of the regression line. However, the use of this approach can contribute to eliminate some uncertainties as to a clear separation between clean and contaminated sites. In view of this, in order to have a statistically meaningful regression, it is necessary to have a high number of samples [36, 37].

The systematics of environmental classification is obtained starting from the CFs obtained for each contaminant in the environment or in the organisms.

When evaluating CFs, it is important to consider all uncertainties due to sampling, space, and time variability of the samples, age, and condition index of the organisms, etc.

The various elements of the uncertainty of measurement in the different steps (sampling, pretreatment, analytical steps) must be duly evaluated within the framework of the approach that has been adopted for the study.

Generally, two approaches are used for the evaluation of uncertainty:

1. the theoretical "bottom-up" approach recommended by international organizations. This method requires the evaluation, expressed as standard deviations, of all factors that will contribute to the final value (e.g. volumetric flask corrections, standard weight corrections, pipet volume corrections, signal uncertainty, etc.).
2. the practical "top-down" approach from the relative standard deviation derived from an interlaboratory study by the Harmonized IUPAC/Association of Official Analytical Chemists (AOAC) protocol [38].

The formal definition of uncertainty given by the ISO Guide [39] is as follows: "Parameter associated with the result of a measurement, that characterizes the dispersion of the values that could reasonably be attributed to the measurand."

Recently, Conti *et al.* [38] analyzed theoretical and practical aspects of uncertainty in environmental laboratory analysis.

In general, a minimum level, below which it is not possible to talk about a certain contamination, is a CF that is higher than a given amount (generally 1.5, 2, or 3 times higher than BL). The qualification of a situation of contamination can follow a linear scale. However, it can also happen that, in the presence of a high level of pollution, there is an exponential scale. Table 2, for instance, shows the contamination situation that can occurs in relation to the CFs of estuary ecosystems.

This is a useful means to control the impact basal levels of the different ecosystems. It also allows a correct formulation of environmental prevention programs both on a large scale and on an industrial level, like in the Environmental Impact Assessment (EIA) studies. As for instance before the building or modification of

Table 2: Concentration factors and contamination of estuary ecosystems.

CF	Situation
<2	negligible
2–6	possible-moderate
6–18	certain-severe
18–54	very severe
>54	critical

big industrial plants (refineries, thermal power plants, steelworks, etc.) or large infrastructure works (public buildings, motorways, etc.).

8 Levels of organization

In short, in order for an organism to be considered a valid bioindicator, it must have a wide geographical distribution, be available all year round, and be also very tolerant to the contaminant (without being killed by it), be easy to sample and store, and, more importantly, it must have a positive correlation between the contaminant concentration accumulated in its organism and the concentration of that same contaminant in the surrounding environment [2].

Table 3: Organization levels and measures connected with biomonitoring studies [15].

-individual -organism -genetic mutations -reproductive success -physiology -metabolism -oxygen consumption, photosynthesis rate -enzyme/protein activation/inactivation -hormones -growth and develop -resistance to disease -tissue/organ damage -bioaccumulation
-population -survival/mortality -sexual rate -abundance/biomass -behavior (migration) -predation rate -decrease/increase population
-community -organism (or organisms) abundance -biomass -organism (or organisms) density -abundance (variety), number of species, width class, or other functional -groups, per area or volume, or number of individuals
-variety/relative abundance of species
-ecosystem -mass nutrients

The identification of cosmopolite indicators allows a comparison between the absolute levels of some contaminants (e.g. metals) of organisms belonging to different geographical areas. Bioindicators are a very valid tool because they can accumulate contaminants such as heavy metals from the aqueous medium up to tens of thousands of times as much as BLs.

Measurements associated with biomonitoring are possible at various levels of organization: individual, population, community, and ecosystem. A synthesis of these by now commonly used methods is reported in table 3.

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References

[1] Conti M.E. (2002) Gli indicatori ed i marcatori biologici in Ecotossicologia. Università degli Studi di Roma, in: Facoltà di Farmacia (Ed.), La Sapienza. Centro Stampa d'Ateneo, Rome, pp. 124–141.

[2] Conti M.E., Cecchetti G. (2001) Biological monitoring: lichens as bioindicators of air pollution assessment – a review. Environmental Pollution, 114, 471–492, Reprinted (or higher parts taken) with a kind permission of Elsevier.

[3] Connell D. (1999) Introduction to Ecotoxicology. Blackwell, Oxford, UK, pp. 184.

[4] Tessier A., Campbell P.G.C., Bisson M. (1980) Trace metal speciation in the Yamaska and St. François Rivers (Quebec). Canada Journal Earth Science, 17, 90–105.

[5] Krumgalz B.S. (1989) Unusual grain size effect on trace metals and organic matter in contaminated sediments. Marine Pollution Bulletin, 20(12), 608–611.

[6] Herman R. (1987) Environmental transfer of some organic micropollutants. Ecological Studies, 61, 68–99.

[7] Bero A.S., Gibbs R.J. (1990) Mechanisms of pollutant transport in the Hudson Estuary. The Science of the Total Environment, 97/98, 9–22.

[8] Moriarty F. (1999) Ecotoxicology: The Study of Pollutants in Ecosystems. Academic Press, London, pp. 350.

[9] Zaghi C., Conti M.E., Cecchetti G. (2002) White paper on chemicals and Stockholm convention on persistent organic pollutants: perspectives for environmental risk management. International Journal of Risk Assessment and Management, 3(2–3–4), 234–245, Reprinted (or higher parts taken) with a kind permission of Inderscience Publishers.

[10] European Commission White Paper (2001) Strategy for a Future Chemicals Policy, COM, 88 final.

[11] Directive 67/548/EEC relating to the classification, packaging and labelling of dangerous substances as amended, directive 88/379/EEC and new directive 99/45/EC relating to the classification of dangerous preparations, directive 76/769/EEC relating to restrictions on the marketing and use of certain dangerous substances and preparations, regulation (EEC) 793/93 on evaluation and control of risks of existing substances.
[12] European Commission Working Document SEC (1998)1986 final.
[13] Stockholm Convention on Persistent Organic Pollutants (2001) URL: http://www.cham.unep.ch/pops.
[14] Carballeira A., Carral E., Puente X., Villares R. (2000) Extensive control of litoral contamination. Nutrients and heavy metals in sediments and organisms on Galicia coast (NW Spain), in: Conti M.E., Botrè F. (Eds.), The control of marine pollution: current status and future trends. International Journal of Environment and Pollution, 1–6, 534–572.
[15] Conti M.E. (2002) Il monitoraggio biologico della qualità ambientale. ed. Seam, Rome, p. 185.
[16] Hertz J. (1991) Bioindicators for monitoring heavy metals in the environment, in: Merian E. (Ed.), Metals and their Compounds in the Environment, VCH, Weinheim, 221–232.
[17] Tonneijk A.E.G., Posthumus A.C. (1987) Use of indicator plants for biological monitoring of effects of air pollution: the Dutch approach. VDI Ber, 609, 205–216.
[18] Stöcker G. (1980) Zu einigen theoretischen und metodischen. Aspekten der Bioindikation, in: Schubert R., Schuh J. (Eds.), Methodisce und theoretische grundlangen der bioindikation (Bioindikation 1), Martin-Luther-Universität, Halle (Saale), GDR, pp. 10–21.
[19] Phillips D.J.H. (1977) The use of biological indicator organisms to monitor trace metal pollution in marine and estuarine environments. A review. Environmental Pollution, 13, 281–317.
[20] Phillips D.J.H. (1980) Quantitative Aquatic Biological Indicators: Their Use to Monitor Trace Metal and Organochlorine Pollution. Applied Science Publishers, London, p. 488.
[21] Slobodkin L. (1961) Growth and Regulation of Animal Populations. Holt, Rinehart & Winston, New York.
[22] Hunt E.G., Bischoff A.I. (1960) Inimical effects on wildlife of periodic DDD application to Clear Lake. California Fish Game, 46(1), 91–106.
[23] Bacci E., Gaggi C. (1998) Bioconcentrazione, bioaccumuloe biomagnificazione, in: Vighi M., Bacci E. (Eds.), Ecotossicologia. UTET, Torino, pp. 143–152.
[24] Renner R. (2002) The K_{ow} controversy. Environmental Science and Technology, 36(21), 411A–413A.
[25] Suedel B.C., Boraczek J.A., Peddicord R.K., Clifford P.A., Dillon T.M. (1994). Trophic transfer and biomagnification potential of contaminants in aquatic ecosystems. Reviews of Environmental Contamination and Toxicology, 136, 21–89.

[26] Focardi S., Leonzio C., Fossi C., Rosi C., Marsili L. (1991) Delfinidi spiaggiati lungo le coste toscane e laziali: livelli di idrocarburi clorurati. S.IT.E. Atti, 12, 197–200.
[27] Focardi S., Marsili L., Fabbri F., Carlini R. (1990) Preliminary study of chlorinated hydrocarbon levels in Cetacea stranded along the Tyrrhenian coast of Latium (Central Italy), in: Evans P.G.H., Aguilar A., Smeenk C. (Eds.), European Research on Cetaceans, Cambridge 4, pp. 108–110.
[28] Fowler S.W., Buat-Menard P., Yokoyama Y., Ballestra S., Holm E., Nguyen H.V. (1987) Rapid removal of Chernobyl fallout from Mediterranean surface waters by biological activity. Nature, 329(6134), 56–58.
[29] Nonnis Marzano F., Triulzi C. (2000) Evolution of radiocontamination in the Mediterranean Sea in the period 1985–1995. International Journal of Environment and Pollution, 13(1–6), 608–616.
[30] Bros W.E., Cowell B.C. (1987) A technique for optimizing sample size (replication). Journal Experimental Marine Biology Ecology, 144, 63–71.
[31] Conti M.E., Tudino M.B., Muse J.O., Cecchetti G.F. (2002) Biomonitoring of heavy metals and their species in the marine environment: the contribution of atomic absorption spectroscopy and inductively coupled plasma spectroscopy. Research Trends in Applied Spectroscopy, 4, 295–324, Reprinted (or higher parts taken) with a kind permission of Research Trends.
[32] International Organization for Standardization – ISO Guide 33. (2000) Uses of certified reference materials.
[33] International Organization for Standardization – ISO Guide 35. (2006) Reference materials – General and statistical principles for certification.
[34] International Organization for Standardization – ISO Guide 32. (1997) Calibration in analytical chemistry using certified reference materials.
[35] Templeton D.M., Ariese F., Cornelis R., Danielsson L.-G., Muntau H., Van Leeuwen H.P., Łobiński R. (2000) Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches (IUPAC recommendations). International Union of Pure and Applied Chemistry, 72(8), 1453–1470.
[36] Conti M.E., Cucina D., Mecozzi M. (2007) Regression analysis model applied to biomonitoring studies. Environmental Modeling Assessment DOI: 10.1007/s10666-007-9113-7 (*in press*).
[37] Conti M.E., Iacobucci M., Cecchetti G., Alimonti A. (2007) Influence of weight on the content of heavy metals in tissues of *Mytilus galloprovincialis* (Lamarck, 1819): a forecast model. Environmental Monitoring Assessment DOI: 10.1007/s10661-007-9875-z (*in press*).
[38] Conti M.E., Muse J.O., Mecozzi M. (2005) Uncertainty in environmental analysis theory and laboratory studies. International Journal of Risk Assessment and Management, 5(2–4), 311–335.
[39] Guide to the expression of uncertainty in measurement (GUM). ISO, Geneva, 1993 (Reprinted 1995). Italian version: Ente Nazionale Italiano di Unificazione (UNI). UNI CEI ENV 13005. Guida all'espressione dell'incertezza di misura, Milano, UNI, 2000.