BIOINDICATORS IN ENVIRONMENTAL MONITORING: BIOLUMINESCENT BACTERIA, ALGAE AND HONEYBEES

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ABSTRACT

Environment protection actions can result effective and adequate when based on a complete awareness about the extension, intensity, quality and respective quantity of chemicals involved, fate during time, and above all about the real impact on the biosphere of the pollution to be removed or to make harmless.

Sophisticated analytical methodologies can offer a multianalyte, traces detections, but a monitoring plan requires the collection of a high number of samples and rapid, low-cost screening analyses before to employ them.

To this aim biosensors and bioindicators can be very useful tools. Among the several organisms which can be employed as bioindicators we have along research experience about luminescent bacteria, microalgae, and honeybees [1-2].

Luminescent bacteria started to be employed for environmental monitoring in the middle eighties and now their use in sediment and water analysis is recognized officially by European and international policies as a reference toxicity test. We performed various monitoring activities [1] by using a bioluminescent bacteria (BLB) based assay, performed at room temperature, rapid and versatile even unspecific. The use of 96 wells microplates allowed to test several sample at one time, to reduce the working volumes and therefore the cost per assay. The analytes were heavy metals, BTEX in aqueous samples, pesticides, hydrocarbons contaminated soils during bioremediation, wastewater effluents from industrial and urban treatment plants.

The BLB test was also associated to microalgae cultures, in order to evaluate the heavy metals (cadmium and copper) bioaccumulation capacity of the microalgae *Pseudokirchneriella subcapitata*, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum*. In most cases solutions from algal cultures had higher luminescence than control heavy metal solutions, demonstrating the bioaccumulation properties.

Honeybees in environmental monitoring were employed first to control the presence of pesticides in the agro-ecosystems [2]. The monitoring techniques, the tests carried out (chemical analysis and pollen identification), the processing of data (through the Index of Environmental Dangerousness) concurred to characterize the areas and the periods of higher contamination, the pesticides the most used (also those whose employment is not allowed) and the treated crops. Later on the bees have been used for the monitoring of heavy metals pollution in cities and industrial areas, as well as to reveal the presence of radionuclides. Moreover bees have been employed for the detection of the phytopatogen *Erwinia amylovora*, coupled to a PCR-ELISA diagnostic technique for the automated detection in pollen

Keywords: Environmental monitoring, bioluminescence, microalgae, honeybees

1. Introduction

Men's activities continuously affect environment at different levels by introducing increasing amounts of known and new kinds of polluting substances.

Traditionally, the impact of pollutants discharged into the environment by human activities has been assessed using chemical assays or evaluating physical parameters. The large variations both in time and space of pollutants' concentration would require a high density of sampling points and/or number of monitoring devices to drawn a realistic picture of the environmental contamination. The high costs derived from such a kind of monitoring activities always limited the number of collected and analyzed samples.

Moreover, it is an accepted assumption that the simple measurement of chemicals concentration, with reference to established regulatory rules, will not give an accurate account of their environmental noxiousness.

The use of living organisms as indicators for environmental quality has long been widely recognized. Over the past few decades, organisms from different trophic levels have been used: cultured cell, bacteria, algae, nematodes, cladocerans, amphiphods, fishes, plants. According to their ecological distribution they were employed as bioindicators and biomonitors in air, soil and water pollution surveys (Gerhardt, 1999; Wolterbeek 2002). These techniques provide information on the response of living organisms to the integrated effects of environmental conditions and of contaminants, and has the advantage of indicating the real impact of all chemicals present in a given sample or ecosystem. Rapid biological tests are playing their major role in hazard and risk assessment, especially at the screening level.

Biomonitoring procedures may allow a detailed and reliable coverage of the territory with relatively low costs and it can be defined as "passive" or "active": passive monitoring is performed through observations and analyses of organisms which are a natural component of the ecosystem, active monitoring includes all methods which insert organisms under controlled conditions into the site to be monitored. In this view, the availability of a large number of reliable bioindicator species is an important step towards a wider application of the methodology (Iriti, 2006)

The luminescence based bioassays for the ecotoxicological assessment of environmental pollutants have been used since several decades ago, and the main tested species included *Vibrio fischeri, Vibrio harveyi* and *Pseudomonas fluorescens.* The luminous bacteria are ubiquitously distributed, the major part being marine forms, free living in water or parasitic to marine or terrestrial animals.

Bioluminescent organisms, naturally or genetically modified to display this feature, free or immobilized to prepare a biosensor probe, can offer the possibility to perform rapid, sensitive, reproducible and cost-effective assays for toxicity screening and assessment in water, sediments, and soils, also offering the further advantage of an easy record of the effects produced on the living organism light emission. Tests can be based on light emission inhibition or induction, depending on the employed organisms. A bioluminescence (BL) inhibition assay is often chosen as the first screening method in a test battery, based on speed and cost considerations. The most employed one is the naturally bioluminescent marine bacterium *Vibrio fischeri*, which application started in the 1970s (Trott, 2007). The bioluminescent bacteria (BLB) tests protocol is usually simple, especially when applied to aqueous samples or extracts: the bacteria emit light when they find themselves in optimal conditions whereas in presence of noxious substances their luminescence decrease. Thus the presence of toxic molecules, as pesticides, heavy metals, organic and inorganic compounds, can be evaluated (Girotti, 2008). Foe their use in toxicity assessment procedures several commercial kits are currently available. The BLB assays can be used both for short- and long-term tests. Short-term (acute toxicity) tests measure effects at 15-30 min after contact and reveal substances producing an immediate, strong effect on bacterial viability. Long-term (chronic toxicity) tests (24 h or longer) examine change in viability or growth rate produced by low concentration or slow-acting pollutants.
Microalgae are the primary producers at the base of the aquatic food chain. They are one of the first groups to be affected by contamination and therefore they provide important information for predicting the environmental impact of pollution in water bodies. Because of their short response times and their sensitivity to a wide range of toxicants at environmentally relevant concentrations, algae often provide one of the first signals of ecosystem impacts, thereby

allowing for corrective regulatory and management actions to be taken before other undesirable effects occur. Algal tests are generally sensitive, rapid and cost effective and are based on the measurement of physiological (growth and photochemistry) or biochemical (enzyme activity, oxidative stress response) changes as test end points (Stauber, 2000)

The inhibition of photosynthesis is an acute end point that can be determined by measuring changes in chlorophyll-a fluorescence. The growth inhibition parameter requires longer assay periods. Algae can be easily cultured at the lab, and the most common species used are both marine and sweet water green microalgae and diatoms.

Honeybees have been identified as a good biological indicator since the beginning of last century, because they detect and reveal the chemical impairment of the environment they live in, mainly through two signals. One is the most evident, i.e. the changes in the mortality, while the second one is represented by the residues collected from the environment on or within their bodies or in beehive products and that may be evaluated by suitable analyses (Porrini, 2002). The effectiveness of *Apis mellifera L.* as an environmental monitor is founded upon several ethological characteristics, among which can be mentioned that:

- Its body is covered with hairs, which makes it particularly liable to hold the materials and substances it comes into contact with.
- It shows a high sensitivity to several compounds, mostly phytopharmaceuticals.
- It shows great mobility and a flying range that allows a vast area to be monitored.
- It displays high efficiency in ground surveys (numerous inspections per day).
- It samples almost all environmental components: soil, vegetation, water, air.
- It brings and stores into the hive nectar, pollen, honeydew, propolis and water.

The management costs are extremely low, especially in proportion to the large number of samples that may be taken.

More recently, a protocol to standardize the use of honeybees as bioindicator has been developed, defining the fundamental units of this activity, the “monitoring stations”, constituted by two beehives, which can be distributed on the whole territory under control on the basis of the ethological characteristics of the bees and of the ecology of the territory itself, creating an high efficient monitoring network.

2. Results

2.1. Bioluminescent bacteria

Water samples represent the easier environmental matrix to be tested by bioluminescence assays. Our experience in the application of *V. fischeri* and *Vibrio sp*. based assays in environment protection activities concerns several aspects, starting from the monitoring of marine and surface water total toxicity levels, in combination with complementary assays (physico-chemical analytical techniques) into a single integrated, remote sensing monitoring platform able to supply in continuous data about the chemical composition and biotoxicity levels in river, lake or marine coastal waters and to release an alarm when one of the monitored parameters changes significantly.

On the other hand, to monitor the presence of heavy metals like Cd and Cu in sea water the BLB were coupled to other organisms, the mollusk *Scapharca inaequivalvis*. The toxicity of the heavy metals collected from sea water by the mollusk and contained in its extracts was evaluated by the BLB assay, creating a complete bioindicators system to monitor coastal pollution.

In a similar way, the toxic impact of a large number of pesticides collected by bees during their gathering activity in the environment and extracted from their bodies, was evaluated by the inhibition of bacteria light emission, behind their identification and quantification by GC techniques.

The pollution produced by the release to the environment of veterinary antibiotics through the manure dispersion on the fields was also evaluated by the inhibitory effects of the antibiotic residues on the bioluminescence of *V. fischeri*.

The residual toxicity in effluents from treatment plants and the efficiency of their various compartments must be continuously monitored. We applied the BLB inhibition assay to obtain those informations about a wastewater treatment plant of a dairy factory and to check the effluents quality of an urban wastewaters treatment plant.

Remediation or bioremediation processes would need a long monitoring period to assess their effectiveness, and low costs for this activity is usually desired.

The evaluation and monitoring of a bioremediation treatment on soils contaminated by hydrocarbons from an oil refinery was another, but not less important, application of the bioluminescent bioassay. Finally, the complex, real impact on the biosphere of the photodegradation products of chemically resistant organic compounds still present in urban wastewater treatment plants (antibiotics, psychotropic drugs, personal care and UV protecting products) has been studied starting from their toxicity on BLB.

2.2. Algae

The microalgae *Pseudokirchneriella subcapitata*, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* were employed both to assess their response to the presence of Cd and Cu ions in solution and their property to bioaccumulate these heavy metals. Algae were put into contact with different concentration of the heavy metal for 72 hours. The solutions were then tested by the BLB assay and the differences in light emission evaluated after 30 minutes. In most cases solutions from algal cultures had higher luminescence than control heavy metal solutions, demonstrating the bioaccumulation properties of the three algae. The EC_{50} values have been determined both for algae and for luminescent bacteria. Among the three kinds of algae the strongest effect was produced by the sweet water green microalga *P. subcapitata* the bioaccumulation of Cd ions resulted higher than that of the Cu ones. In addition, some algal samples showed an unknown feature that enhances the BLB light emission, an interaction which deserves further studies.

2.3. Honeybees

Since 1998 we undertook various monitoring projects with bees in order to identify any cases of pesticide abuse or misuse. Different analyses have been used for the qualitative and quantitative determination of the compounds extracted from bee’s body or beehive products (gas chromatographic analysis, enzymatic and immunoenzymatic methods). Coupling these data with a palinological analysis is possible to create a map of pesticide distribution on the territory.

The reliability of bees and honey for the monitoring of heavy metals (Pb, Ni and Cr) was assessed. Fresh honey was finding to be the best matrix to use in the environmental monitoring programs, because of the average information it is able to supply as it comes from the nectar collected for several days in large areas.

Bees were successful in detecting radionuclides when applied to monitor radioactive emissions from a nuclear fuel processing plant or, following Chernobyl accident, in monitoring the distribution of long life nuclides, such as ^{137}Cs and ^{90}Sr in hive products and in several environmental compartments. The measurements of beta and gamma activity showed that pollen was the most efficient indicator of atmospheric radionuclide contamination as it reflects that of the air. Bees have proved to be highly effective in detecting even very low levels of environmental radioactivity, as it occurred during the release of ^{137}Cs at Algeciras (Spain). The levels of radioactivity were many times below any alarm threshold, but the bee matrix promptly revealed its presence in the atmosphere.

Furthermore, bees could be used as a bioindicator to detect the presence of phytopathogenic microorganisms. A study was conducted on *Erwinia amylovora* – causal agent of a severe disease among *Rosaceae* known as “Fire Blight”. Bees were responsible to transport this microorganism from infected to unaffected trees. By developing an original analytic method based on PCR-ELISA and immunoenzymatic determination with chemiluminescent detection we were able to demonstrate the possibility of detecting the presence of *E. amylovora* in an area considered still unaffected, using bees to keep under continuous surveillance large areas for long periods. Several months later the disease devastatingly manifested itself in that area we previously indicated under risk.

3. Conclusions

The various biosensors we used during our researches demonstrated their suitability and sensitivity in detecting the different pollutants. Nevertheless, it is acknowledged that the “battery of test” approach, utilizing several different short-term biological tests, would be preferred in any monitoring scheme, since the specific or unspecific sensitivity of an organisms to pollutants represents just its answer to it, not all the possible effects on the whole biosphere

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