ANALYTICAL TECHNIQUES AND BIOINDICATORS IN ENVIRONMENTAL CONTROL: HONEYBEES, MUSSELS, BIOLUMINESCENT BACTERIA: RAPID IMMUNOASSAYS FOR PESTICIDE DETECTION

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Abstract. The toxic pollutants are widely distributed on terrestrial and marine environment and their early identification is fundamental to prevent or to control the damages to humans and environment. The environmental monitoring of toxic pollutants by bioindicators like bioluminescent bacteria (BLB), mussels and honeybees, both in terrestrial and marine environment is reported. BLB and mussels have been employed to assess the toxicity of heavy metals in lagoon slime samples and seawater, respectively. Honeybees were applied, coupled to suitable immunoassays, to the determination of azinphos-methyl and thiram pesticides.

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1. Introduction

The use of living organisms as indicators to determine the environment quality has long been widely recognised. Over the past few decades plants, animals, fungi, and bacteria have been employed as bioindicators and biomonitors in air, soil and water pollution surveys (Alfani et al., 1996; Gerhardt, 1999; Nimis et al., 1990; Henderson, 1996; Wolterbeek, 2002).

Biological monitoring may be defined as the measurement of the response of living organisms to changes in their environment and it can be divided into "passive" and "active". Passive monitoring is performed through the observation and analysis of organisms which are usual inhabitants of an ecosystem; active monitoring includes all methods which insert organisms, under controlled conditions, into the site to be monitored. In general, (Ceburnis and Valiulis, 1999) these techniques provide information on the response of living organisms to the integrated effects of environmental contaminants, which cannot be determined by direct analytical measurements. Biomonitoring may allow a detailed and reliable coverage of the territory with relatively low costs (Girotti et al., 2006a) and, in general, is based on sensitive or accumulative organisms, i.e. bio-indicators or bioaccumulators (Batzias and Siontorou, 2007).

Bioindicators display a very high sensitivity to pollutants, providing information that is function of the quality of the environmental sector under examination (Butterworth et al., 1995). They can give information on: (1) decrease of the biotic diversity with a decrease of the species constituting the community and an increase of individuals belonging to few, resistant species; (2) presence or absence of a particular species; for example, *Plecoptera* can live only in cold water rich in oxygen; (3) appearance of a structural damage, reversible or permanent.

An organism, to be employed as a bioindicator, has to have the following characteristics: to be easily identified; to be easily sampled; to be widely spread in the studied area; to have a low mobility; to have a long-life cycle; to have a good genetic uniformity on all the considered area; to be present all-year long. Bioindicators permit to establish a network of monitoring sites with a consequent satisfactory coverage of the territory without the requirement of sophisticated and expensive equipments and power. 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 et al., 2006). In addition, data obtained by biomonitoring are not related to a single sampling, but they are the summa of very high number of sampling.

An organism is defined "bioaccumulator", when the modality of distribution of pollutants can be inferred thanks to their concentrations in the organism (Batzias and Siontorou, 2007). The accumulative species can store contaminants

in their tissues, since bioaccumulation is an equilibrium process of biota compounds intake/discharge from and into the surrounding environment. The disadvantage to employ bioaccumulator organisms is the need for sometimes complicated analytical techniques and equipments to determine the compound of interest.

Since several years the Analytical Chemistry Section of SMETEC focuses its research activity on the development of assays based on luminescent reagents, as well as on the environmental monitoring by bioluminescent bacteria (BLB), mussels and honeybees, employed as bioindicators (Girotti et al., 2002a, 2005a, 2006a, b).

The bioassays based on luminescence have been used for several decades, and test species include *Vibrio fischeri, Vibrio harveyi* and *Pseudomonas fluorescens* (Girotti et al., 2001). Between them, the most employed one is the naturally bioluminescent marine bacterium *Vibrio fischeri*, the use of which was developed in the 1970s (Trott et al., 2007). The bacterial assays are usually rapid, reproducible, cheap and in some case can replace tests using animals, resulting very useful to measure the toxicity of different kinds of environmental samples (Parvez et al., 2006). The toxicity test is usually based on the bioluminescence inhibition assay: the luminescent bacteria emit light when they find themselves in optimal conditions, whereas in presence of noxious substances their luminescence decreases. Thus, the presence of toxic molecules, as pesticides, heavy metals or organic compounds can be evaluated (Girotti et al., 2002b). The luminescent bacteria system can be applied both as short- and long-term test (El-Alawi, et al., 2002). Even if short test has some obvious advantages, it has also some not negligible drawbacks, i.e., it can reveal only molecules acting immediately and underestimate the toxicity of chemicals which show mainly long term effects. The bioluminescent bacterial tests can be used both for water and solid samples. In the latter case the tests are applied to elutriates and extractable components, being suitable also for the evaluation of soil bioremediation (Plaza et al., 2005), as reported by Girotti et al. (2007). Recently, Trott et al. (2007) demonstrated as the response of bioluminescent bacterial assays for soils are comparable to those of higher organism and they can represent a valid complementary tool to traditional methodologies, when an accurate validation and an optimised extraction protocol have been obtained.

One of our more recent applications of the BLB tests is to the analysis of sludge and soil contamination and bioremediation (Girotti et al., 2005c). During sludge remediation treatments, for example on a proper boat, the biotoxicity of the materials and the effects of the treatment must be frequently evaluated, and such a control can be easily performed even in these conditions by BLB toxicity assays. This kind of assay is already widely employed and it has been standardized as an ISO procedure (BS EN ISO 11348-3, 1999).

Mussels are recognized as pollution bioindicator organisms because they accumulate pollutants in their tissues at elevated levels in relation to pollutant availability in the marine environment. Moreover, this ability has led to the adoption of the international "Mussel Watch Project" and several national programs on Mussel Watch in the marine environment have been carried out (Jernelov, 1996). Mussel is widely used as sentinel organism for the assessment of persistent organic pollutants contamination, heavy metals, organotin compounds in freshwater environments, polycyclic aromatic hydrocarbons, DDT bioaccumulation (Kramer, 2006; Binelli et al., 2006).

During a study performed at the laboratories of the Center of Water Biology, Institute of Experimental Zooprophylaxis of Abruzzo and Molise (Italy), the bivalve molluscs *Mytilus galloprovincialis* has been inserted in a device designed for the continuous monitoring of water bodies, the Mosselmonitor, and a model system, sea water contaminated by heavy metals (cadmium, lead, copper and mercury) has been used to evaluate the sensitivity of the biosensor, which resulted in a range useful to detect real pollution levels.

Pesticide residues in agriculture lands require to be continuously monitored because of their toxicity to human health and the potential hazard for the conservation of the ecological equilibrium. Pollinators, specially honeybees, are considered a reliable biological indicator (Bromenshenk et al., 1995) because they reveal the chemical impairment of the environment by the high mortality and retaining suspended particles in the air or present on the various parts of the plants. It is possible to collect the dead honeybees by means of traps and then to identify the gathered cultivation and area through pollen analysis. It is possible to univocally determine the origin of a certain pesticide by means of a net of beehives. Besides pesticides, heavy metals and radioactive elements have been monitored by analyzing the residues on honeybees body (Porrini et al., 2002).

Azinphos-methyl, [*O*,*O*-dimethyl *S*-[(4-oxo-1,2,3-benzotriazin-3(4*H*)-yl) methyl] phosphorodithioate], is an organodithiophosphorus insecticide and acaricide introduced in 1954, and it was proved to be very toxic to honeybees. Its analysis is generally performed by liquid-solid extraction followed by gas chromatographic techniques (Mercader and Montoya, 1999).

Thiram (tetramethylthiuram disulfide) (bis (dimethylthiocarbamyl) disulfide) is a dithiocarbamate, a wide-spectrum, non-systemic, fungicide, applied on humans against scabies, as a sunscreen agent or as a bactericide in soaps (Sharma et al., 2003).

Immunochemical techniques have gained an increasing importance for screening and quantification of pesticides due to their sensitivity, speed, simplicity, and low cost (Dankwardt, 2001). The enhanced chemiluminescent (CL) reaction offers the possibility to improve the sensitivity of immunoassays compared to conventional colorimetric detection (Botchkareva et al., 2003).

We developed a sensitive indirect chemiluminescent enzyme-linked immunosorbent assay (CL-ELISA) (Figure 1) that employs luminol, horseradish peroxidase as labeling enzyme, based on monoclonal antibodies (MAbs) (Mercader and Montoya, 1999) for the detection and quantification of azinphosmethyl in honeybees (Girotti et al., 2005b), and a CL-ELISA based on polyclonal antibodies for the detection and quantification of thiram in honeybees that allow rapid and sensitive screening of large numbers of samples (Eremin et al., 2006).
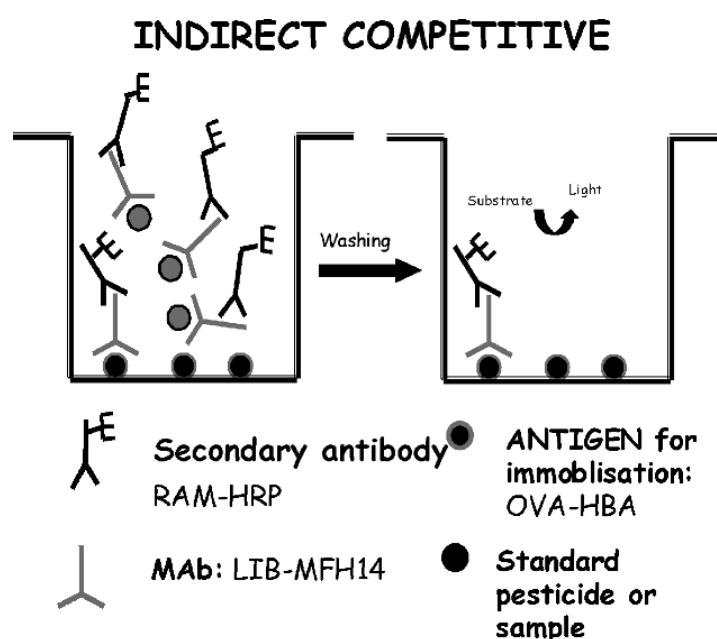

INDIRECT COMPETITIVE

Secondary antibody
RAM-HRP

MAb: LIB-MFH14

ANTIGEN for
immoblisation:
OVA-HBA

Standard
pesticide or
sample

Figure 1. General scheme for an indirect competitive ELISA.

2. Material and Methods

2.1. BIOLUMINESCENT BACTERIA

Three different bioluminescent bacteria were used, two belonging to the species *Vibrio logei*: Ucibo and Russian, the first isolated from water of the Mediterranean Sea, the second one supplied by the Institute of Biophysics (Siberian Branch, Academy of Sciences), Cultures Collection IBSO, Laboratory of Bacterial Bioluminescence. Akademgorodok, Krasnoyarsk (Russia). The third was the *Vibrio fischeri* strain. Bacteria have been cultivated and employed, working at room temperature, as reported previously (Girotti et al., 2002b, 2007).

The BL analyses were made on a set of eleven samples (including that one designed as "blank") of sediments collected by the Taverna SpA (San Giorgio di Nogaro (UD), Italy) at different locations in the lagoon of Marano, Grado, and in the Aussa-Corno river, areas of intensive industrial settlement. Detailed description of all locations and the geological and physical characteristics of the samples were previously reported (Coletti et al., 2006).

The samples were divided into three groups, according to the sampling place: the Marano Channel group (samples 0078, 0080, 0108, 0114), the Aussa Corno Banduzzi group (samples 0904 and 0906) and the Foce Aussa Corno group (samples 1077, 1094, 1110 and 1119). All results were compared with the values of the "blank". This was a sample showing geological characteristics similar to the samples and harvested in a zone considered free from pollution.

Samples were extracted with water or ethanol (1 g in 5 mL) and the extracts diluted with a NaCl 2% solution to avoid osmotic problems to the marine bacteria. Luminescent measurements were done using a Wallac "Victor 1420" Multilabel Counter luminometer (Wallac, Sweden) and employing 96 wells microplates. The inhibition assay was performed by adding to each well 100 µL of the solution containing 3% of the extract and 100 µL of bioluminescent bacteria in culture broth. The emitted light was recorded, at intervals of 10 minutes, for 30–60 min in short-term assays and for 16–24 hours in long-term assays. The light emission of the bacterial suspension was tested before to start the assays in order to check if the intensity was optimal for the analysis.

2.2. MUSSEL MONITOR

The evaluation of mussels monitoring of sea water polluted by heavy metals was done at the Center of Water Biology, Institute of Experimental Zooprophylaxis of Abruzzo and Molise "G. Caporale", Termoli, Italy. The preparation of standard solutions and set up of the Mosselmonitor were done as previously reported (Barile et al., 2007). Briefly, the sea water introduced in the device was withdraw from the same area of the sea where the mussels naturally grow, about 500 m from the Adriatic coast in front of Termoli, Molise (Italy), and mussels have been employed as a group of 8 individuals, placed inside the Mosselmonitor (Figure 2).

The instrument Mosselmonitor has been supplied by Delta Consult (The Netherlands). The system is based on the measurement, by electromagnetic induction, of the distance between the two valves by means of a couple of coils sticked on the valves. The intensity of the measured electric tension is inversely proportional to the distance between the coils, indicating the position of the two valves.
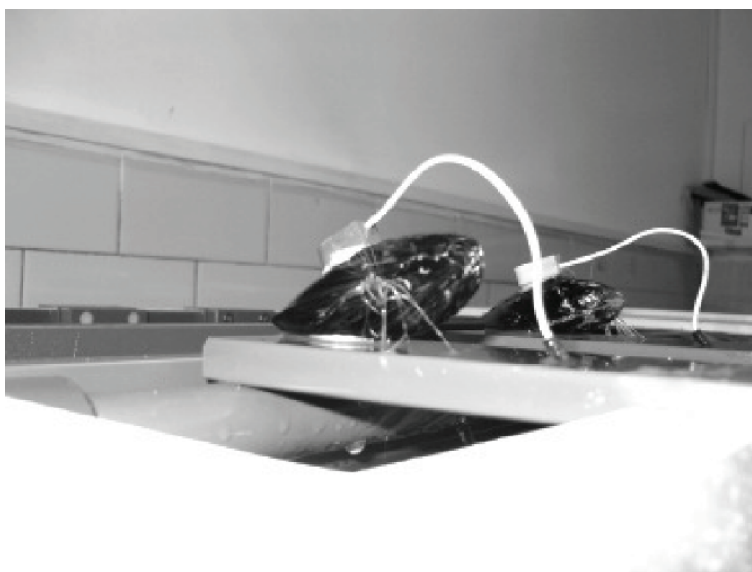
Figure 2. Mussels in the Mosselmonitor device.

The sea water chemical-physical parameters (temperature, pH, oxygen and salts concentrations) have been checked each two hours by a multi parametric equipment. The whole water volume (200 L) circulated among the instrument tank and two more tanks by a pumping system, in a closed cycle. A first tank received the water going out from the Mosselmonitor® tank, from which it was pumped to a second tank where oxygen was bubbled inside. Finally, water returned to the Mosselmonitor tank.

Data produced by mussels shell movements, expressing their wellness or suffering, were collected continuously by a software, that represented them in graphs showing the medium value of the distance between the valves (percentage of valves opening) as well as the time periods of opening and closure of all mussels. Moreover, data about each mussel were available, to as certain individual anomalous behaviour.

The position of the valves was recorded each 90 seconds and the significant changes from the usual behaviour were elaborated and expressed by the instruments as "alarms" (Kramer and Foekema, 2000). The "A" type alarms express the hyperactivity of the mussels, which open and close the shell at high frequency. This behaviour reveals a condition of deep suffering of the organisms and it is very reliable. The "C" type alarm appears when a certain number of organisms remain closed for a time period longer than usual. In this study a period of 15 min has been defined as the minimum to get a "C" alarm. The "D" type of alarm is produced when the filtration activity is reduced, i.e., the average percentage of opening is reduced during a certain period of time.

Unfortunately this condition occurs not only in presence of pollutants but also when some environmental conditions changes, for example the water turbidity. For this reason this alarm is very sensitive but not reliable.

Each 24 h the molluscs were feeded with hypo dietetics rate of algae *Chaetoceros spp* collected from laboratory cultures.

The behaviour of the organisms, after they have been placed inside the Mosselmonitor, has been observed for 1 month, then the whole volume of water was changed and the toxicity test was started by adding increasing concentrations of each heavy metal. The test was divided into two phases. First, the organisms were left to stay for 36 h after the water was changed, their behaviour was observed for 12 h more and then the toxicant solution was added. After this, the alarms generated by the pollutant effects were recorded for 24 h. The concentrations were chosen in a range based on the Quality Standards defined for the year 2008 by the EU Legislation (European Community, 2000). Suitable amounts of stock solutions were added to the first tank to obtain, in the whole volume of 200 L, the following concentrations: 0.2, 10, 40, 80 and 100 ppb for cadmium; 125, 250, 500, 1000 and 2000 ppb for lead; 2.5, 5, 10, 20 and 40 ppb for copper; 0.01, 0.03, 0.5, 1 and 5 ppb for mercury. Bioluminescent measurements on the same heavy metals solutions have been performed by the Microtox kit and the luminometer Microtox MD500 (Ecotox, Italy), equipped with a cell thermostated at $15 \pm 1°C$, at the wavelength of 490 nm.

2.3.   HONEYBEES FOR AZINPHOS-METHYL AND THIRAM IMMUNOASSAYS

Honeybees samples were collected, stored and prepared as previously described (Ghini et al., 2004). To analyze the pesticides we applied two kind of extraction: the classical liquid-liquid extraction (acetone-dichloromethane with purification by phosphate precipitation) and a new graphitized carbon extraction; the extracts were simply diluted prior to use (Ghini et al., 2004).

Standard pesticides were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). Peroxidase-labeled goat anti-rabbit immunoglobulins were from Dako (Glostrup, Denmark). All chemicals and organic solvents used were of reagent grade or better. Black polystyrene high-binding plates were from Costar (Cambridge, USA). Before analysis, honeybee samples were lyophilized and stored at -5°C.

Azinphos-methyl immunoassay: The Chemiluminescent azinphos-methyl immunoassay has been previously developed (Girotti et al., 2005b). Briefly, the plates were coated overnight with 100 µl of the appropriate concentration of the heterologous OVA-HBA conjugate in coating buffer (50 mM carbonate/bicarbonate, pH 9.6) (Girotti et al., 2005b). The plates were then washed four times with washing solution (0.15 M NaCl with 0.05% Tween 20). A volume of

50 µL/well of standard in bidistilled water or sample solution, followed by 50 µL/well of LIB-MFH14 MAb were added. The competitive reaction was allowed to take place for 1 h. Inhibition standard curve was prepared by serial dilutions from 500 to 6.40 x 10^3 nM with a dilution factor of 5. After washing, 100 µl of a 1/2000 dilution of peroxidase-labeled rabbit anti-mouse immuno-globulins in PBS (10 mM phosphate, 137 mM NaCl, 2.7 mM KCl, pH 7.4) was added, and plates were incubated for 1 h. Plates were then washed and finally peroxidase activity was revealed by adding 100 µL/well of a freshly prepared substrate mixture (1 mM luminol, 0.5 mM p-iodophenol, 1 mM H_2O_2 in 0.2 M borate buffer, pH 8.5). Intensity of chemiluminescence emission (RLU) was measured by the "Victor 1420" microplates reader (Wallac, Finland), immediately after the addition of the substrate mixture.

Thiram immunoassay: The starting basis of the CL thiram immunoassay was the colorimetric ELISA for thiram already reported by Queffelec et al. (2001). Assays were performed in 96-well microplates as indirect competitive format (Eremin et al., 2006). Plates were coated overnight with 100 µL/well of OVA-hapten 2C (0.6 µg/mL) in carbonate-bicarbonate buffer 0.05 M pH 9.6, which corresponded to 0.06 µg/mL of OVA-hapten-2C per well and then washed three times with 0.01 M, pH 7.4 PBS supplemented with 0,05% Tween 20, as washing solution and after addition of 50 µL/well of standard or sample plus 50 µl/well of antiserum (1/30 000 in 2x fish gelatine solution in PBS) were incubated for 1.5 h at room temperature. After washing, 50 µl/well of per-oxidase-labeled goat anti-rabbit immunoglobulin, diluted 1/2 000 in 1x PBS-Gelatine, were added and incubated again for 1.5 h at room temperature. After washing, 100 µL/well of a substrate solution (1 mM luminol, 0.5 mM p-iodophenol, 1 mM H_2O_2 in 0.2 M borate buffer, pH 8.5) were added and intensity of chemiluminescence emission, expressed as relative light units (RLU), immediately measured in a "Victor 1420" microplate luminometer (Wallac, Finland).

RLU values from standards were mathematically fitted to a four parameter logistic equation (Botchkareva et al., 2003). The limit of detection (LOD) for ELISAs was calculated as the analyte concentration that reduced signal to 90% of the maximum. The extracts were also analyzed by gas-chromatography (Ghini et al., 2004; 2005b).

3.   Results and Discussion

3.1. BIOLUMINESCENT BACTERIA

Quantitative analysis of the samples content of heavy metals were done to characterize them, and an example is showed in Figure 3.
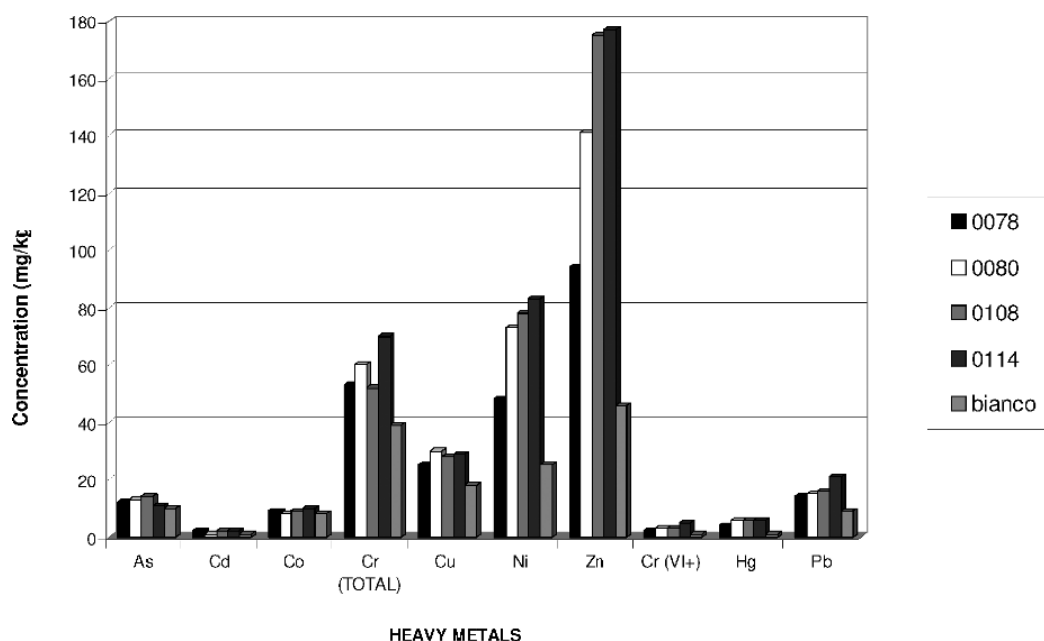
Figure 3. Heavy metals content in some of the analysed sludge samples.

To evaluate the limits of detection of the bioluminescent assay, that have to be lower than the Maximum Admitted Limits according to the regulatory rules, standard solutions of each heavy metal present in the sludge samples were analysed both by the short and the long term assays and the assays showed a linear behaviour in different ranges of concentration.

As reported in Table 1 the BBL assay showed good sensitivity and the limit of detection (LOD) and EC_{50} values were lower than the maximum admitted levels.

TABLE 1. Limits of detection and EC_{50} values of six heavy metals, obtained for the BLB assay, compared with their legal limits

Heavy metal	LOD (ppm)	EC_{50} (ppm)	Legal limits in surface water (ppm)	Legal limits in wastewater (ppm)
Pb (II)	0.1	0.2	<0.2	<0.3
Hg (II)	0.005	0.05	<0.005	<0.005
Cd (II)	0.03	0.5	<0.02	<0.02
Zn (II)	1	3.0	<0.5	<1
Cr (VI)	0.2	5.0	<2	<4
Cu(II)	0.2	0.3	<0.1	<0.4

The extraction of heavy metals from the samples, made by water or by 2% NaCl solution gave almost the same result, in the meaning that the inhibition curves obtained by using the two extracts are very close, practically overlapped (Figure 4).
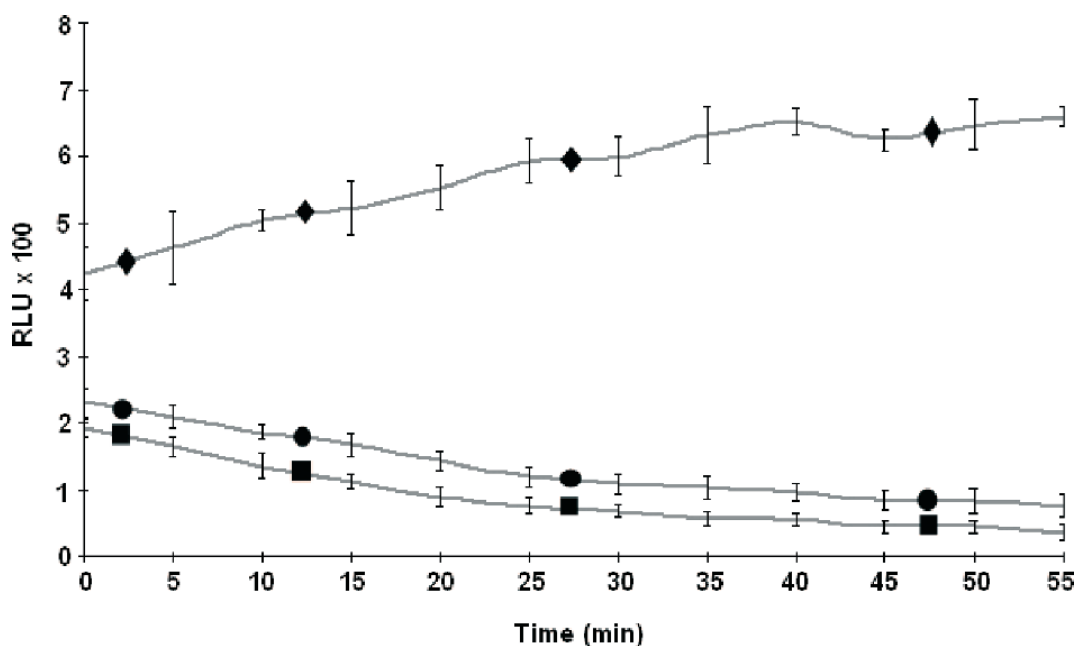

Figure 4. Acute inhibitory effects of the water and 2% NaCl extracts of the sample 1119 on the Ucibo light emission. ■ 1119 2%NaCl; ● 1119 water + 2%NaCl; ♦ Blank.

The HPLC analysis demonstrated that the "blank" sample was contaminated with low concentrations of heavy metals, and these low amounts did not cause a significant bacterial luminescence decrease.

The samples have been analysed by all three BLB strains for the acute and chronic toxicity (Figure 5), both at short and long term, showing different influence on the different strains.

The sample extracts showed different effects also when emitted light intensity was compared with the "blank" emission. In fact, the light emission from the different strains in contact with the same extract resulted in one case inhibited, in another one stimulated, with respect to the "blank". As shown in Figure 6 the major part of the samples inhibited the emission of *Vibrio fischeri*, while the same produced a hormesis effect (Christofi et al., 2002) on the Russian strain, probably because this strain was able to use some compounds in the sample extracts as a substrate. These differences were observed both at short and long term. The importance to test the same samples by different BLB strains is clearly confirmed by these results.
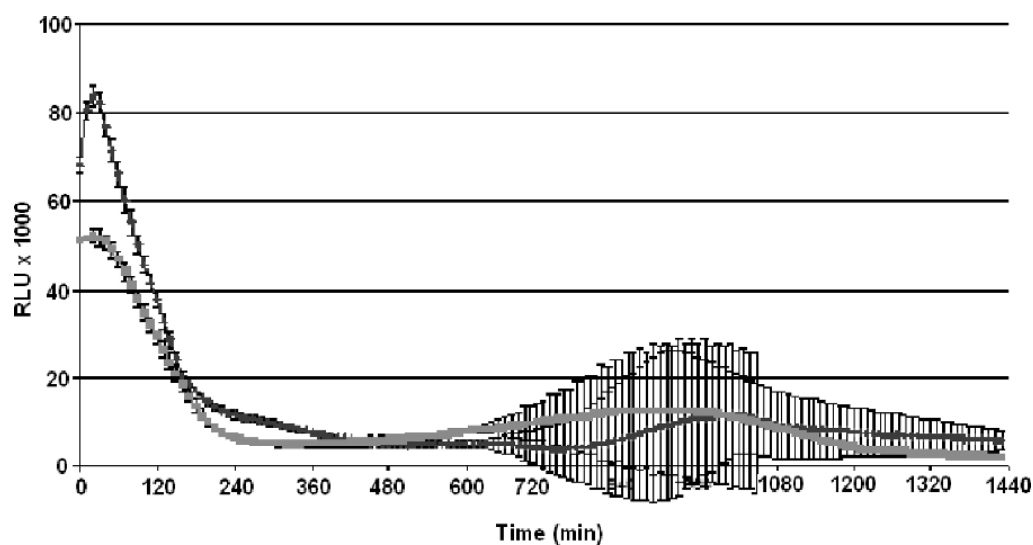
Figure 5. Example of chronic BLB assay, black line: blank; gray line: 0080 sample.

In the following Table 2 the respective behaviour of the three strains with respect to all samples have been summarized.

TABLE 2. Differences of the response of the various strains to the addition of the sample extracts

Sample/ Bacteria	78	80	108	114	904	906	1,077	1,094	1,110	1,119
Vibrio	T	T	A	A	T	A	A	A	N	A
Russian	T	N	T	T	A	N	A	A	T	N
Ucibo	N	N	T	T	A	T	T	T	T	T

The behaviour of the three bacteria strains, in presence of the samples where: T – typical behaviour (the bioluminescence curve in presence of the sample in below to that of the black). A – a typical behaviour (the biolumine-scence curve in presence of the sample is above that one of the black). N – the bioluminescence curves are not distinguishable.

In this kind of analyses it is very important to have the possibility to measure several replicates of the same sample, to ensure reproducible results. For example, we determined that in long-term assays it is advisable to measure at least five replicates for each sample, to obtain a CV (coefficient of variation) in reasonable limits (under the 15%). By using the microplate format this number of replicates can be prepared and measured at the same time without difficulties, since small amounts of reagents are required.
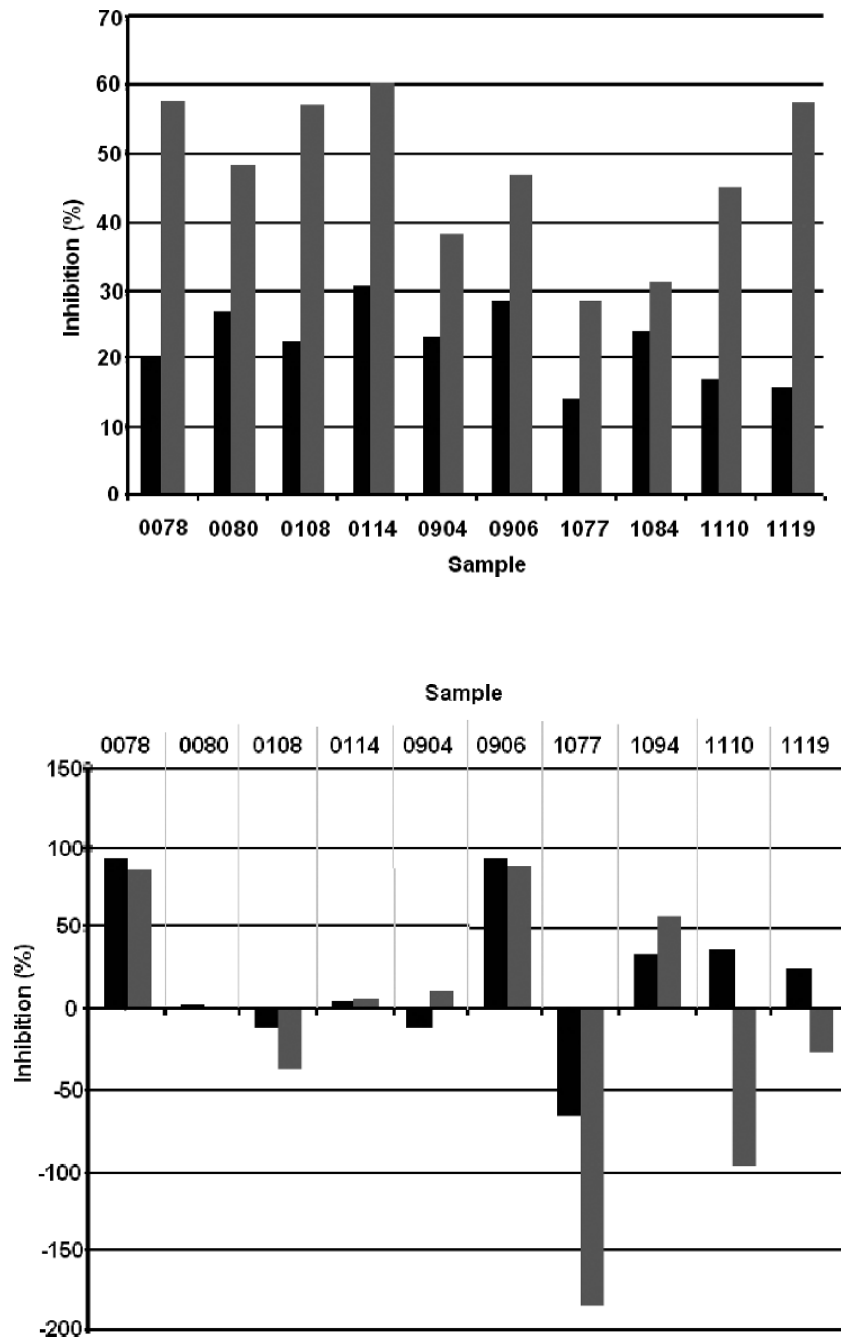
Figure 6. Acute (black) and chronic (gray) BLB assay, % inhibition of lagoon samples on Vibrio and Russian strain.

3.2. MUSSEL MONITOR

To estimate the toxicity of various concentrations of Cu, Cd, Pb, and Hg the measured parameter was the maximum length of the C alarms recorded during the period of contact with the toxicant (Figures 7–10).
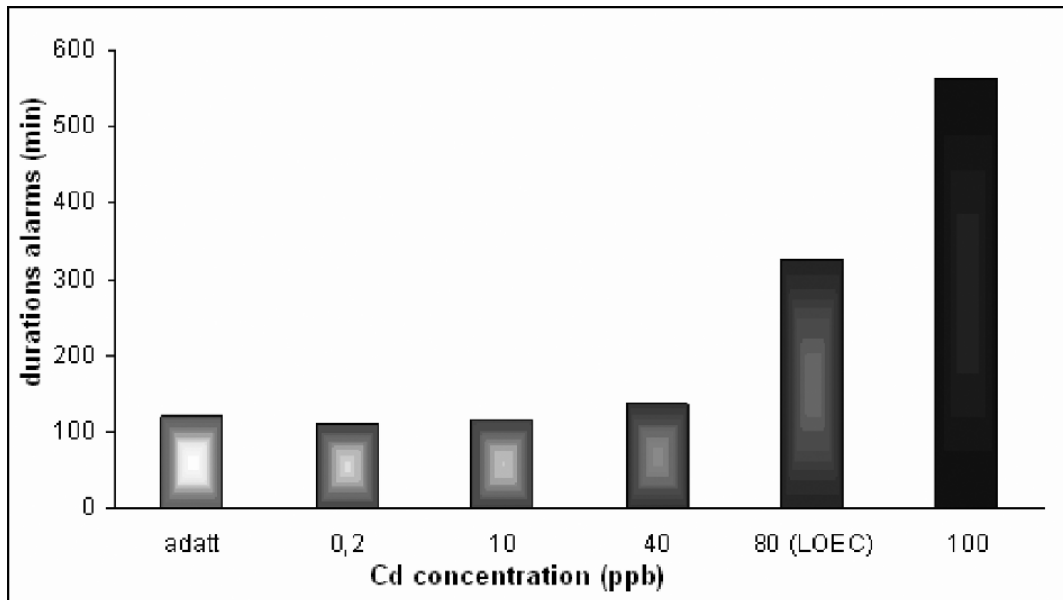

Figure 7. Maximum length of the C alarms corresponding to each one of the tested concentration of cadmium.
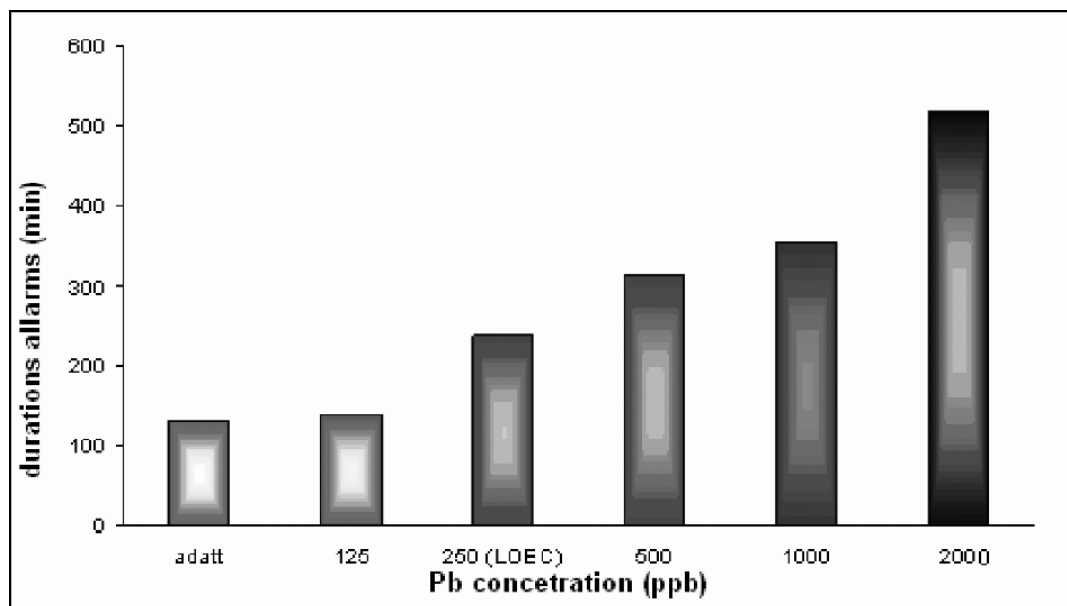

Figure 8. Maximum length of the C alarms corresponding to each one of the tested concentration of lead.
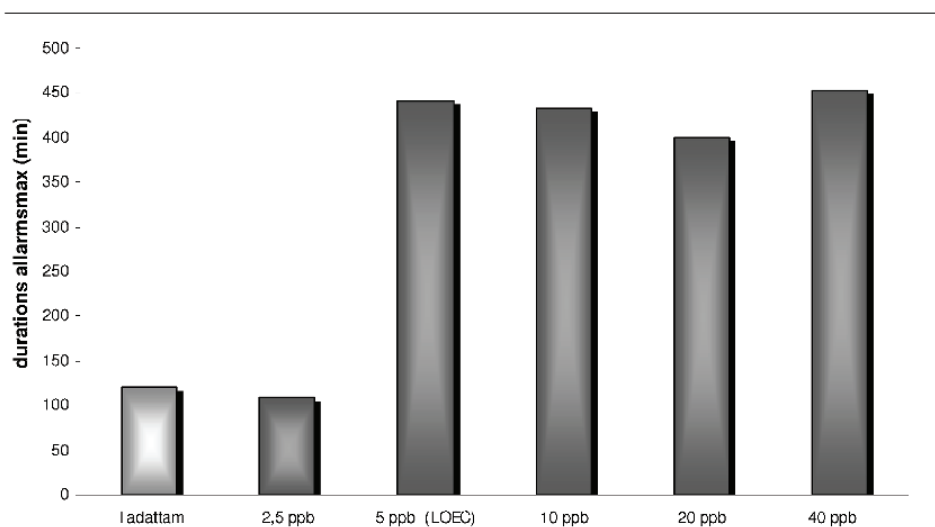
Figure 9. Maximum length of the C alarms corresponding to each one of the tested concentration of copper.
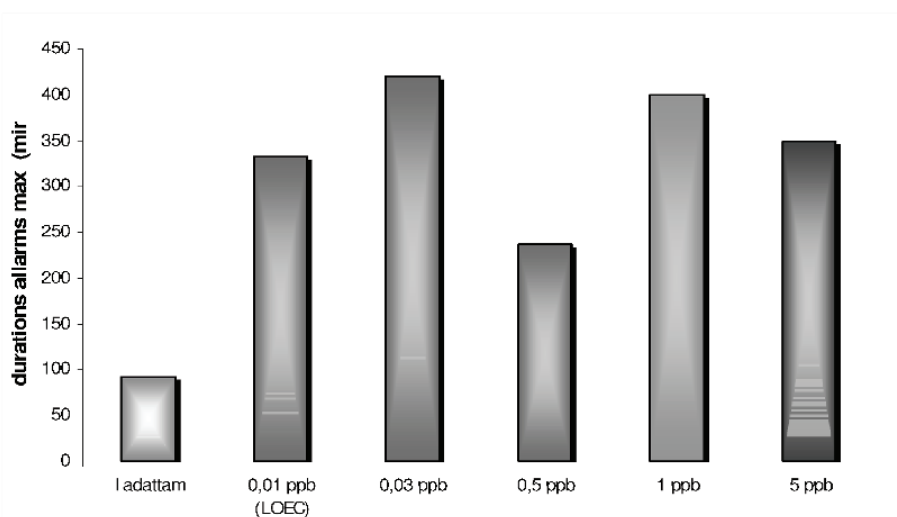

Figure 10. Maximum length of the C alarms corresponding to each one of the tested concentration of mercury.

It is possible obtain, for each metal, the duration of the longer alarm clearly different from the adaptation period and the corresponding concentration was defined as the Lowest Observed Effect Concentration (LOEC) (Kramer and Foekema, 2000). The LOECs obtained were: 5 ppb for copper, 0.01 ppb for mercury, 80 ppb for cadmium and 250 ppb for lead. The performances of the biosensor were compared with the response of a widely employed test for acute toxicity, the Microtox® kit, based on marine bioluminescent bacteria *Vibrio fischeri*. The luminescent test resulted less sensitive, with LOEC of 1, 0.5, 10 and 1 ppm, respectively.

Both the instrumental and the biological components showed the required characteristics to obtain a suitable biosensor. Concerning the bioluminescent assay the comparison with the mussel biosensor demonstrated that, even the first is very simple and widely employed, it is sensitive only at high concentration of the tested pollutants.

3.3. HONEYBEES AND PESTICIDES

3.3.1. *Azinphos-Methyl Immunoassay*

The ELISA was optimised and characterised by determining several parameters. Immunoreagents optimum concentrations were determined by bidimensional titration in the range 2–0.2 µg/mL for OVA-HBA and 60–7.5 ng/mL for LIB-MFH14. The ability of the chemiluminescent technique to detect lower concentrations of HRP allowed the optimal antibody concentration (30 ng/mL) to be decreased, compared to colorimetric (COL) ELISA for Azinphos-methyl, and the hapten-protein conjugate concentration was selected at 1 µg/mL for CL-ELISA) (Mercader and Montoya, 1999) (Table 3). The highest sensitivity was obtained for 60 min incubation.

TABLE 3. Comparison of the analytical performance of the CL ELISA and the colorimetric one

ASSAY	CONJUGATE ng/mL	ANTIBODY Dilution	ng/mL			
			IC_{50}	IC_{20}	IC_{80}	IC_{90}
CL-ELISA-	1.0	1:30000	88.5	100	71	39
COL-ELISA by ALP*	60	1:30000	34.0	90	11	5
COL-ELISA by HRP*	60	1:30000	35.0	110	11	5

*ALP = alkaline phosphatase; HRP = peroxidase enzymes.

Four organic solvents, methanol, ethanol, acetone, and dioxane, to be used for Azinphos-methyl extraction were assayed for their effects on the immunoreagents in the range 0–10%. Solvents were generally well tolerated, with only slight variations of IC_{50} and RLU max. Optimized assay conditions allowed to obtain, on standard solutions in solvents, the IC_{50} value of 0.40 nM, and a limit of detection of 0.01 ng/mL. A typical competitive curve for Azinphos-methyl is shown in Figure 11.
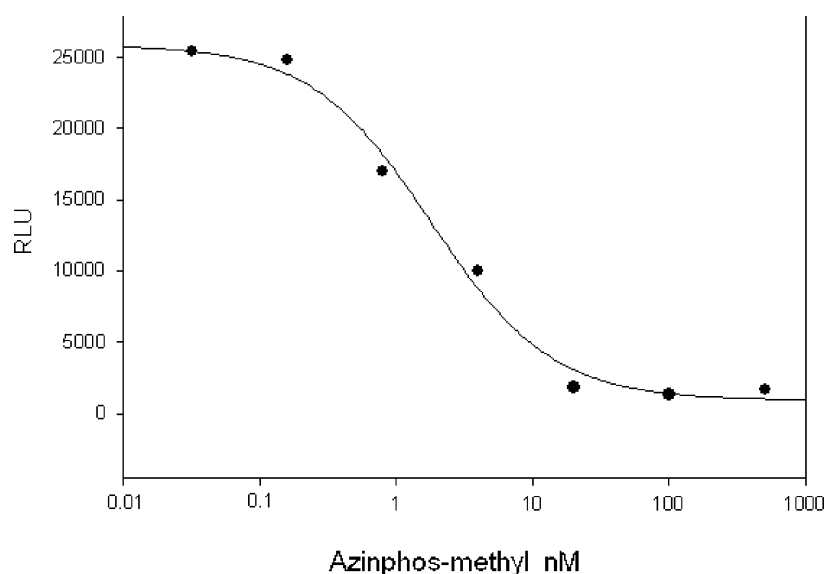
Figure 11. A typical competitive curve for Azinphos-methyl.

To study matrix interferences, standard curves were also prepared in extracts from non contaminated honeybees. Two different extraction procedures were tested: the liquid-liquid extraction and a graphitized carbon-based solid-phase extraction. A very important matrix effect was encountered: undiluted samples, as well as 1/5 and 1/50 dilutions greatly disturbed ELISA assay, while the 1/100 dilution allowed to obtain only slight decrease of the maximum emission, with minor increase of IC_{50}.

The non contaminated honeybee samples were spiked at different levels (0.1 μg/mL, 1 μg/mL and 10 μg/mL) and then extracted and analysed. The recovery proved to be higher with the graphitized carbon procedure (>60%) than with the liquid-liquid extraction (20–40%). Finally, the assay was applied to the analysis of azinphos-methyl in honeybees samples collected in Russia. 5 samples out of 19 were found positive. For two of them, the presence of azinphos-methyl was confirmed by gas chromatographic analysis, while the concentration of the other samples was smaller than the detection limit of the chromatographic technique (10 ng/mL).

3.3.2. *Thiram Immunoassay*

The assay was optimised and characterised by determining several parameters. Immunoreagent optimum concentrations were determined by bidimensional titrations. The most appropriate work conditions have been obtained using

0.6 µg/ml of OVA-2C (0.06 µg/well), anti-thiram serum dilution of 1/30,000 and peroxidase-labeled goat anti-rabbit immunoglobulin were diluted ½,000. Highest sensitivity, related to the anti-thiram serum incubation time was obtained for 90 min. To assess tolerance to organic solvents, acetone and methanol where added at four different final concentrations: 3%, 5%, 10% and 20%. Comparing IC_{50} values and RLU max for both solvents at each concentration, acetone 3% final concentration assured the optimal conditions for CL-ELISA of thiram. The competitive assay for thiram showed an IC_{50} of 120 ng/ml and a LOD of 20 ng/ml.

Also in this case the honeybee extracts were diluted 100 fold to eliminate the matrix effect. The non contaminated honeybee extracts have been spiked with 0.5 ppm and 1 ppm and the recovery was higher for liquid-liquid extraction, 54%, than for SPE, 31%, underlying the difficulties due to the complexity of the honeybee matrix.

Finally the CL-ELISA applied to real honeybees samples, 5 collected in Italy and 5 in Russia demonstrated that all Italian samples were negatives, and 3 of Russian samples were positive.

Conclusion

The presented BLB test can be used to evaluate the toxicity of heavy metals in solid samples also during the remediation procedure, because the decrease of light emission from the bacteria is directly related with the presence of metals affecting the cells viability. The bioluminescence test allows to analyse at the same time, by using the microplate luminometer, a lot of samples in a relatively fast way, at low costs and using small volumes of reagents.

The data obtained on the applicability of the Mosselmonitor – *Mytilus galloprovincialis* biosensor as an Early Warning Alarm System represent interesting, positive indications for the monitoring of sea environment. Nevertheless, it must be remembered that the mussel monitoring procedure must be carried out by personnel with a deep knowledge of the ecological characteristics of the employed organisms, since this is necessary to perform a correct interpretation of the data supplied by the instrument. It must be underlined that neither the LOEC determined by the biosensor, nor the EC_{50} values obtained with the bioluminescent bacteria are in agreement with the Quality Standards levels that must be reached according to the national and international legislation for the year 2008 (European Community, 2000). Those levels could be probably obtained by employing a set of organisms at different trophic levels (Dalzell et al., 2002), and among them the mussels can be surely included.

The chemiluminescent immunoassay developed for the detection and quantification of Azinphos-methyl allowed performances comparable to those of the colorimetric assay, but utilising a smaller amount of monoclonal antibodies.

The assay was applied to the analysis of honeybee samples, proving to be suitable for the use in environmental monitoring programs.

The competitive CL-ELISA developed for the detection of thiram in honeybees showed great specificity and a quite good sensitivity. The analysis of thiram in honeybees samples posed several problems due both to its instability and to the complexity of the matrix. The important matrix effect produced by the extracts can be overcome by a 100 fold dilution, that allowed to obtain reproducible results, although this resulted in a lighter decrease of the sensibility. It is important to underline that the developed chemiluminescent assay, using specific antibodies, is able to detect the thiram as itself and not as one of its degradation products, and that it is a semi quantitative method that allows a rapid and easy screening of honeybee samples.

Acknowledgements

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