BIOSENSORS: NEW FRONTIERS FOR THE ENVIRONMENTAL ANALYSIS

Katia Buonasera, PhD[1]; Gianni Pezzotti, PhD[1,2]; Ittalo Pezzotti, PhD(c)[1,2]; Juan Bernado Cano PhD(c)[1]; Maria Teresa Giardi PhD[1]

[1]Istituto di cristallografia-Consiglio Nazionale delle Ricerche Via.Salaria Km 29,3-00015 Monterotondo(Rome)
[2]Biosensor srl - Via degli Olmetti 44 -00060 Formello (Rome) Italy

**Corresponding at the author:* Researcher IC-CNR[1] e-mail: katia.buonasera@mlib.ic.cnr.it

ABSTRACT

Biosensors are very promising biotools useful for the fast, simple, cheap and reliable screening of many real samples. For their intrinsic features, these devices can find application in many different fields, but appear particularly useful for those requiring repeated analyses daily performed, such as biomedical, agricultural and environmental fields. This article reviews the biosensors which appeared useful for environmental analysis. In particular, the work focused on photosynthesis-based biosensors, i.e. biosensors whose biorecognition elements are represented by whole cells, or parts of cells, able to photosynthesize. An overview on this type of biosensors, included the methods to immobilize the biomediators and detect the pollutants, is given here along with the most recent examples of their application.

Keywords: Biosensors, PSII-based biomediators, electro-optical transduction systems, pre-screening analysis, herbicide detection

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BIOSENSORES: LA NUEVA FRONTERA PARA EL ANALISIS AMBIENTAL

RESUMEN

Los biosensores son bioherramientas muy prometedoras, útiles para la detección rápida, sencilla, económica y confiable de muchas muestras reales. Por sus características intrínsecas, estos dispositivos pueden tener una aplicación en muchos campos diferentes, pero parecen ser especialmente útil para aquellos que requieren análisis repetidos diariamente realiza, tales como campos de la biomedicina, la agricultura y el medio ambiente. Este artículo revisa los biosensores que parecía útil para el análisis del medio ambiente. En particular, el trabajo se centró en los biosensores basados en la fotosíntesis, es decir, los biosensores, cuyos elementos de biorreconocimiento están representados por células enteras o partes de las células, capaces de realizar fotosíntesis. Una visión general sobre este tipo de biosensores, incluidos los métodos para inmovilizar los biomediadores y detectar los contaminantes, se da aquí, junto con los más recientes ejemplos de su aplicación.

Palabras claves*: biosensores, PSII basado en biomediadores, sistemas de transduccion electro-opticos, analisis pre-screening, detección de herbicida*

1. INTRODUCTION

The detection and quantification of analytes has always been an issue of particular concern in all existing areas, such as clinical diagnostics, food technology and environmental monitoring. Thanks to the rapid bloom and growth of biosensors, bioanalysis has advanced at vertiginous rates. Unlike sophisticated analytical techniques, biosensors lead to easy, fast and low-cost methods to detect and quantify analytes in real time.

A biosensor is an analytical device that consists of a biorecognition element in intimate contact with a transducer element (see fig. 1).

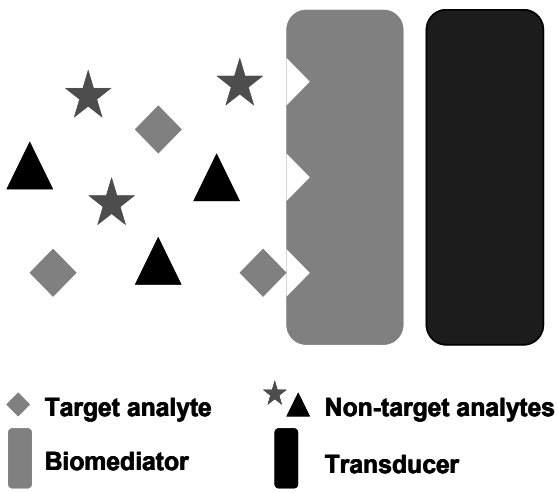

Fig. 1 Generic representation of the components of a biosensor system showing the specificity of the biomediator for some particular analytes and the direct contact of the biomediator with the electronics.

The biorecognition molecule, also known as bioreceptor (or biomediator), is immobilised on the transducer and specifically recognises (via catalytic or affinity interactions) the target. The transducer, in turn, converts the biorecognition event into a quantifiable signal and this combination provides an analysis tool competitive with the established and conventional analysis techniques. Biosensors are classified according to the biorecognition molecule, or the transducer method. (See fig. 2) summarizes the two classifications.

Each one of the techniques used for the signal transduction has advantages and drawbacks. Fluorescence techniques, for instance, although highly sensitive, are characterized by an expensive required equipment. Electrochemical transduction, on the contrary, offers advantages of both sensitivity and relatively inexpensive instrumentation and also the possibility of the instrument miniaturisation, that make this transduction method very attractive for the development of portable devices for *in situ* monitoring. Especially interesting are techniques that do not require labels for the transduction of the binding event, such as piezoelectrochemical detection or surface plasmon resonance (optical technique based on the change in the refractive index of a surface when a biomolecule is immobilised or when an affinity interaction occurs).

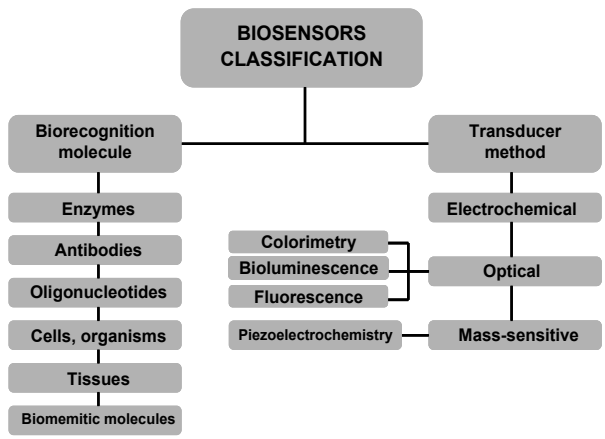

Fig. 2 -General scheme showing how biosesensors can be classified depending on the biomediator or the transduction system used.

Label-free strategies lead to short analysis times and simple operation protocols, and eliminate possible undesirable effects, such as steric impediments, binding biases and instability of the label. However, instrumentation costs and operational requirements of these techniques tend to be elevated, and this fact has strongly limited their achievement.

In general, it can be said that fluorescence and electrochemical techniques represent the most successful ones, in the biosensor field. These techniques are described in a number of articles, where the types of biomediator used vary enormously. This review will only describe biosensors exploiting plant or algae components as biorecognition elements, being these biosensors the most commonly reported. In particular, we will focus on photosynthesis-based biosensors giving a

description for their preparation, their principle of functioning and their use for environmental applications.

Two important experiments have been performed on board Soyuz and Endeavour Shuttle The first, carried on by Photo II device, aimed to detect the effect of radiation on photosynthetic oxygenic microorganisms; the second, performed by Night Vision device, had the purpose of assessing the effect of cosmic rays on a structure present inside *Chlamydomonas. Reinhardtii* algae and mimicking human retina.

Photo II is a device that has been designed and developed within the framework of the Photo Project, as part of the Moma project "From Molecules to Man: Space Research Applied to the improvement of the Quality of Life of the Aging Population on Earth" funded by European Space and Italian Space Agencies [1].

On space flights Photo II measured the chlorophyll fluorescence induction curve in photosynthetic organisms, recorded and stored the data in a flash memory and provided the living conditions essential for the survival of the biological samples, by providing day/night cycles produced by white light emitting diodes (LEDs). Photo II used electronic components specifically designed to withstand Space conditions.

Night Vision has been design to host mutants of algae for the experiment titled "Eyespots and Macular Pigments Extracted from Algal Organisms Immobilized in Organic Matrix with the purpose to Protect Astronaut's Retina".

In one case, that of Photo II, the experiments performed in space stimulated the idea of taking advantage from the device designed for carrying on similar measurements on Earth.

As a result of Space-Earth technological transfer of Photo II, derived fluorescence based biosensors can be developed for application in environmental monitoring (e.g. water pollutants, quality control of drinking water), agriculture and industrial process control.

A biosensor is an apparatus that can detect a biochemical variable using a biological component (tissues, cells, enzymes etc.) interfaced with an electronic transducer. It produces an electrical signal that is easy to process that correspond at the variable being analyzed. The biosensor is characterized by the sensitivity and selectivity of the response of its biological components and by the fact that it is economical, easy to use, of miniature size and versatile [2, 3, 4].

Biosensors have emerged as a promising technology especially in applications where real-time monitoring is required. This technology offers several advantages, since biosensors can be easily used both in laboratory and field applications [3].

Fluorescence biosensors allow simultaneous and multiparametric analyses to be performed, combining the three basic mechanisms of biological recognition: biocatalytic, bioaffinity and cell-based metabolic systems. In particular, among the sensing elements there are photosynthetic microorganisms, part of those, DNA, enzymes, binding-proteins etc. for the detection of several chemical species such as environmental pollution i.e. global toxicity, pesticides, pathogens and heavy metals [3, 5].

Night Vision is also described together with explanation of the importance of using biological organisms in space as models to achieve information which can be transferred on humans. Finally an illustration of Photo II technological transfer is given as a series of biosensors designed and developed for application on the Earth [5, 6, 7, 8].

2. PHOTOSYNTHESIS-BASED BIOSENSORS

Photo Among the biosensors using plant tissues as biomediator, it is worth focusing on those exploiting the photosynthesis. The advantage of using these biosensors is that photosynthesis is highly sensitive to many environmentally important classes of pollutants, such as triazines, phenylureas, diazines, and heavy metals, which today are still widely employed for the weed control in agriculture.

Photosynthesis is a chemical process that converts carbon dioxide into organic compounds (especially sugars), using the energy from sunlight. The process starts with a charge separation in the photosynthetic reaction centre of photosystem II (PSII), an enzymatic chlorophyll–protein complex embedded in the thylakoid membranes of plants, algae and cyanobacteria, which is the real sensing

element of PSII-based biosensors. This charge separation consists in a series of reactions leading to the production of ATP and NADPH, which are the energetic molecules needed for all cellular activities. Most of herbicides inhibit photosynthesis usually by targeting the charge separation, thus blocking the release of ATP and NADPH [1]. These herbicides act by competing with the natural plastoquinone for the binding site in the D1 protein (located in the reaction centre of the PSII) and by avoiding photosynthetic electron transport [2]. Based on this principle of operation, several photosynthetic biosensors have been developed for herbicide monitoring [3]. Most of them utilize intact cells or PSII particles isolated from plants, algae or cyanobacteria to measure either changes in photocurrent [4,5], or inhibition of the electron transport, by means of artificial mediators [6-9]. The choice of using whole cells or sub-particles is simply driven by a matter of choosing between lower efficiency biomediator systems and longer extraction procedures. In general, in fact, using whole photosynthetic cells simplifies the procedure required for the preparation of a biosensor, although the low permeability of the cell membranes to electrolytes is not a negligible problem. On the contrary, using isolated chloroplasts or thylakoids as biological receptors offers a higher sensitivity towards pollutants, due to the direct contact between the functional sites and the operational medium, but in this case the need for additional extraction steps complicates the procedure, making it longer and more laborious.

PSII-based biosensors test the inhibition of the Hill reaction by using artificial electron acceptors, such as 2, 6-dichlorophenolindophenol (DCPIP). Since a major drawback of using photosynthetic material is its short life-time, immobilization techniques are fundamental to increase the stability of the biomaterial. Several techniques have been investigated over the last years. A comprehensive description of them is given below.

2.1 Immobilization techniques

After isolation, the activity of cells and photosynthetic materials undergoes a rapid decrease. In order to preserve their vitality, different physical and chemical immobilization procedures can be employed. Physical methods consist in the adsorption of photosynthetic materials on specific supports, or the inclusion in natural/synthetic gels. In terms of preservation, adsorption certainly represents the best method, allowing the activity of the biological material to remain almost unaltered. However, this simple, economic and mild technique leads to weak interaction forces which cannot avoid the risk of desorption phenomena. This is the reason why immobilization by gel-inclusion, in which the biomediator is entrapped in a three-dimensional polymer network, has been widely employed over the last ten years [3, 10-15]. Chemical methods, originally studied for enzyme immobilization, provide covalent bonds between the biomediator and the immobilizing agent, allowing a more stable interaction during the measurement. Some chemical methods, because of the possible denaturing effect of the binding agent on the photosynthetic material, proved to be unsuitable, so that the choice of the chemical agent requires a more thorough study. Glutaraldehyde (GA) represents one of the chemicals most used in this field. As it has already been demonstrated [16], this cross-linker, which does not affect the Hill reaction activity that leads to oxygen production, allows the immobilization of the photosynthetic material [17] with a slight denaturing effect, provided proteins are added during the phase of polymerization.

As reported in Buonasera et al. [18], for thilakoids extracted from spinaches and whole algae cells, adsorption on specific supports (filter paper discs, alumina filter discs, glass microfibre filters, columns containing diethylaminoethyl-cellulose) and gel inclusion (natural gels: polysaccharides, agar, agarose, carrageneen and alginate; synthetic gels: polyacrylamide, polyurethane prepolymer, photo-crosslinkable resin prepolymer, vinyl monomers, poly(vinylalcohol) polymers, and poly(vinylalcohol) bearing styrylpyridinium polymers an be considered the best physical methods. Among chemical methods, the immobilization by bovine serum albumin-glutaraldehyde (BSA-GA) still appears as the most suitable for amperometric and optical biosensors. The advantage of coupling GA with BSA is that the network of covalent bonds built by GA involves the free $-NH_2$ groups of both the photosynthetic material and the exogenous protein, thus reducing the denaturing effect on the biomediator.

As for the selection of the biomediators, there is not a precise rule for selecting the best immobilization method: that strictly depends on the nature of biomaterial, its resistance to physical/chemical treatments, and its stronger or weaker affinity for the support that must be used.

2.2 Detection systems

Biosensors are generally classified according to the biological material (enzymes, antibodies, oligonucleotides, cells and whole organisms, tissues, biomimetic materials) immobilized on the transducer, or according to the transduction method used to convert the biochemical signal into an electric one. Electrochemical (amperometry, potentiometry), optical (colorimetry, bioluminescence, fluorescence), and mass-sensitive (piezoelectrochemistry) are the most commonly employed transduction techniques in biosensors. In particular, amperometry and fluorescence are reported in most of the papers dealing with photosynthetic biosensors.

Fluorescence. Chlorophyll fluorescence is a sensitive, non-invasive and highly versatile tool by which chlorophyll molecules, excited with a light of appropriate wavelength, return to the ground state emitting light at longer wavelength than that of absorption. In normal conditions, only 1 or 2% of the total light absorbed by chlorophyll molecules of photosynthetic organisms is re-emitted as chlorophyll fluorescence [19]: actually, most of light energy is involved in photosynthesis, while a little amount is dissipated as heat. These three processes, however, compete with each other in such a way that any increase in the efficiency of one, will result in a decrease in the yield of the other two.

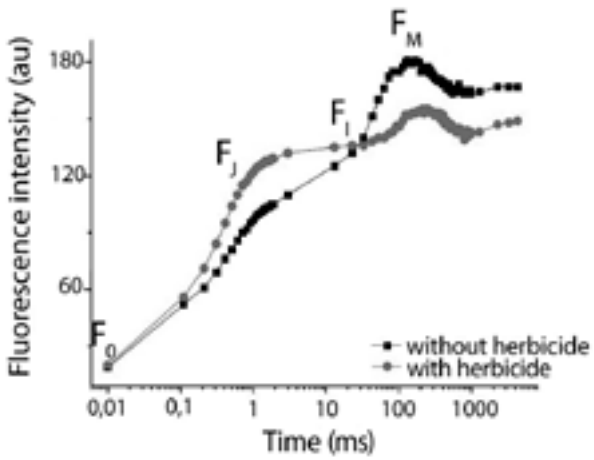

Fig 3. A typical Kautsky fluorescence transient exhibited by dark-adapted *C. reinhardtii* whole cells immobilized on silicon septa, upon illumination by saturating light at 650 nm wavelength. F_0, F_J, F_I and F_M parameters are shown before (dark line) and after (grey line) the addition of herbicide. *(By courtesy of Buonasera et al. [18])*

The presence of herbicides, due to the high affinity of these compounds towards the Q_B site of D1 protein of PSII, fully or partially blocks the photosynthesis by interrupting the electron transfer from Q_A to Q_B plastoquinones. The excess of absorbed light energy, under such conditions, is converted into fluorescence whose yield can be easily measured and which corresponds to the concentration of herbicide.

An example of fluorescence measurement is reported in (see fig. 3) showing the typical Kautsky fluorescence transient curve.

The variations of the parameters described in the curve, and obtained in response to environmental or chemical changes, give information about the electron transport and the photosynthetic metabolism of the biomediator.

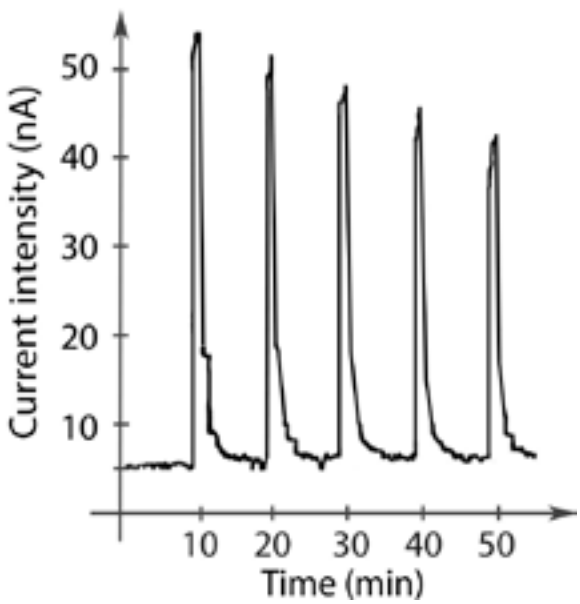

Fig 4. Example of amperometric measurement performed in static mode on thylakoids from *S. oleracea* immobilized with BSA-GA on a screen-printed electrode. Atrazine is added at intervals of 10 min at 4 different concentration (10^{-9}, 5×10^{-9}, 10^{-8} and 5×10^{-8} M) starting from 20 min. *(By courtesy of Buonasera et al. [18])*

3. ENVIRONMENTAL APPLICATIONS

The physiological parameters which describe the photochemistry and the fluorescence emission profile of a photosynthetic organism, are

proportional to the pool size of the electron acceptors Q_A on the reducing side of PSII. The variation of these parameters is symptomatic of the effect of various environmental stresses and indicates a loss of photochemical efficiency. If the electron transfer from the reaction centre to the quinone pool is blocked, such as during the binding of the photosynthetically active pesticides, these parameters change dramatically and this change can be easily monitored by electro-optical biosensors.

The advantage of biosensors based on photosynthesis is that this enzyme complex recognizes certain environmentally important classes of toxic compounds and pollutants commonly found on the surface and in ground waters. These chemicals belong to the classes of (i) phenyl carbamides or ureas or arylureas, such as diuron, neburon, isoproturon, clortoluron, linuron, metobromuron, cicluron, methabenzthiazuron and etidimuro; (ii) triazines, such as atrazine, simazyne, ametryne, desmetryne, prometryn, terbuthylazine, terbutryn, terbumeton, clanazine, metribuzin, metamitron and exazinone; (iii) diazine, such as bromacil, lenacil, cloridazon, piridate and bentazone; (iv) phenols-such as bromoxynil and loxynil [8]. Most of these compounds can bind reversibly to the D1 subunit of PSII within or close to its Q_B binding pocket, altering or inhibiting the electron transfer by displacing the plastoquinone Q_B. As a consequence, electron flow and oxygen evolution are blocked and both current and fluorescence properties of PSII are modified. The variation of these properties is proportional to the concentration of pollutants present, and this gives one the possibility of obtaining calibration curves and performing quantitative analysis. (See fig. 5) illustrates two examples of diagrams showing different herbicides analyzed by fluorescence and amperometry, using two biosensors already on the market: the OPTICBIO-multicell fluorimeter and the AMPBIO-SPE amperometer (Biosensor s.r.l., Rome, Italy, www.biosensor.it).

Several biosensors based on the PSII have been reported as able to detect pesticides in the environment. Euzet et al. 2005 [20], Giardi et al. 2005 [6] and Breton et al. 2006 [21] developed optical biosensors based on the fluorescence activity of thylakoids extracted from various photosynthetic organisms, for the detection of ureas, diazines, triazines and phenolic compounds within a range of recognized concentrations from 10^{-9} to 10^{-5} M. Similar biosensors were projected, implemented and tested, in terms of detection limits, reversibility, and long-term activity, by several research groups**.** Naessens et al. 2000 [22], developed a biosensor based on the chlorophyll fluorescence variation of *Chlorella vulgaris* for the detection of toxic compounds, including diuron, atrazine, simazine, alachlor and glyphosate, with limits of detection between 10^{-3} and 10^{-8}. Marty et al. 1995 [23] employed chloroplast and thylakoids membranes from several organisms for the design of biosensing systems for the detection of photosynthetic herbicides, in addition to enzymes and antibodies as alternative recognition elements for different classes of pesticides, insecticides and organophosphorus compounds.

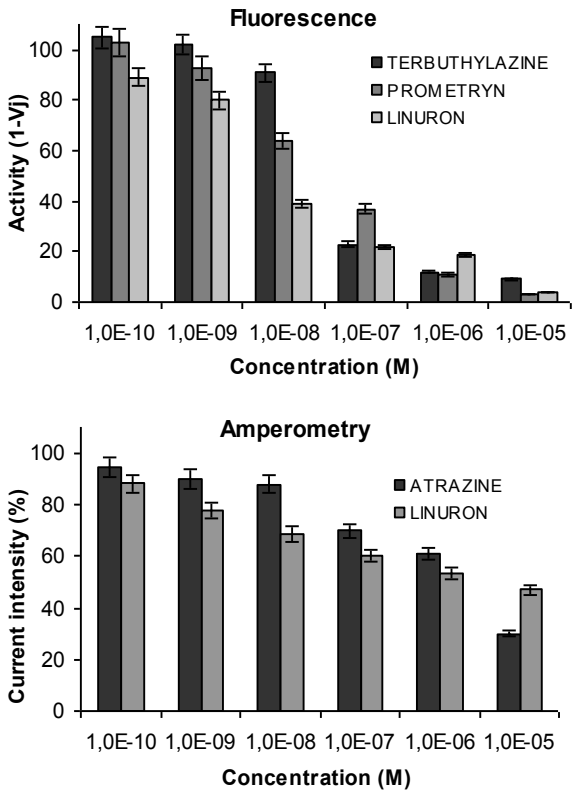

Fig. 5. Diagrams of calibration showing different herbicides analyzed by optical (on the left) and amperometric biosensors (on the right) by using *C. reinhardtii* and spinach thylakoids, respectively *(By courtesy of Buonasera et al. [18])*

These first systems proved to be useful for rapid monitoring of pollutants and environmental pre-screening but the sensitivity was insufficient to reach the concentrations imposed by several

regulations on the environment. The issue of the low sensitivity has been recently overcome by using new biosensors with improved features for signals amplification and conversion. In this context, an optical biosensor based on the green photosynthetic alga *Chlamydomonas reinhardtii* described by Tibuzzi et al. 2007 [15] was employed to monitor several classes of herbicides, such as atrazine, diuron, ioxynil, terbuthylazine, prometryn and linuron, in a lower concentration range (10^{-10} - 10^{-8} M). A multi-biomediator fluorescence biosensor based on a new versatile portable instrument was assembled by Scognamiglio et al. 2009 [11]. It was composed of a 24 cell array configuration able to host different mutant strains from *C. reinhardtii* for the detection of a variety of herbicide classes such as triazines, diazines and ureas. The portable and automatic instrument was equipped with 96 LEDs with different emission peak wavelengths, for fluorescence excitation, and 24 silicon photodiodes and optical filters, for the measurement of the fluorescence emission in response to the presence of the most commonly used herbicides. This biosensor configuration allowed the design of a biosensing system based on an array of engineered biomediators, which showed different sensitivity/resistance, selectivity, and low detection limits (from 1.0 x 10^{-9} to 3.0 x 10^{-10} M) towards a wide range of herbicide subclasses. These biodevices could reduce the analysis complications linked to sample heterogeneity, the wide variations in concentration range and the sample handling and preparation and could be useful for in-field analysis.

In Giardi et al. 2005 [6], for instance, a fluorescence multi-biosensor was reported based on the thylakoids activity from different microorganisms used for the determination of several pollutants on real samples from the Tiber river (RM), the Acqua Marcia (RM), the Valle del Sorbo (Formello), and the Po (FE) river, tested contemporaneously by gas chromatography-mass spectrometry (GC-MS). The water samples were filtered and concentrated for the fluorescence measurements and similar data on the presence of herbicides were obtained in comparing with the standard analytical methods, which showed the absence of simazine, diuron and classical herbicides, while atrazine was present at the level of parts per thousand only in the Tiber and the Po waters. The total amount of herbicides identified by GC/MS was determined by the optical biosensor in terms of inhibition activity. In Touloupakis et al. 2005 [8], the same experiment was performed on an amperometric multi-biosensor, using various photosynthetic biosensing elements for the detection of herbicides and pollutants on real samples. Four river samples were analysed in this study. All the river samples were filtered and concentrated 1000 times with respect to the environmental sample, and finally the water extract were tested using an amperometric biosensor based on the photosynthetic activity of thylakoids extracted from *S. oleracea* immobilized by cross-linking onto a BSA-glutaraldehyde matrix and entrapped in gelatin. The current profile of the biological material was registered in the presence of a river sample flow and the thylakoids activity was analysed and compared with data obtained by GC-MS/MS Ion-Trap technique, showing a perfect correspondence and therefore the usefulness of biosensors for environmental analysis.

4. CONCLUSION

Biosensors will rapidly become essential analytical tools, since they offer higher performance in terms of sensitivity and selectively than any other currently available diagnostic device. A large number of biosensors have already been developed in research laboratories and the corresponding literature in this area is considerable. Biosensors have been designed for many different purposes, but those intended for environmental analysis showed a great potential for future environmental monitoring programs. Although high performance liquid chromatography (HPLC) and gas chromatography (GC) (mainly if coupled to mass spectrometry (MS)) are still considered the techniques of choice, for routine analysis, when hundreds of samples must be daily screened, biosensors represent a useful, easy and fast way to drastically reduce the number of tests. By performing an in-field pre-screening of samples, in fact, those that result “negative” can be discarded while considering only the positive ones for further laboratory investigation.

The attention paid from all over the world to the concern of pollution, there is a considerable need to project and realize devices with features of high selectivity, sensitivity, stability, reproducibility and low cost. With appropriate progress, biosensors can satisfy these requirements along with the advantage of reduced costs for maintenance and personnel.

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