

New Platform of Biosensors for Prescreening of Pesticide Residues To Support Laboratory Analyses[†]

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Millions of tons of pesticides are applied worldwide annually in agriculture. Among them, herbicides such as triazines and ureas, originating from agricultural runoff, can contaminate soils and surface and ground waters with severe toxic effects on humans. Nowadays, different analytical techniques are available for the detection of these chemicals; however, most of them are expensive and time-consuming, especially in the case of routine analyses. For this reason, on the basis of results collected through many years of experience in the field of photosynthetic organisms, we designed a biosensor platform intended for the easy, low-cost, and fast prescreening of photosynthetic herbicides. The platform combines the possibilities of amperometric and optical transduction systems, which have proven to be highly sensitive (limits of detection = 10^{-10} – 10^{-8} M). The use of genetically modified algae strengthens the power of the platform, allowing different subclasses of herbicides to be recognized. The system has been validated for the analysis of environmental water and is proposed to support laboratories involved in the control of water pollution.

KEYWORDS: Biosensor platform; PSII-based biomediators; electro-optical transduction systems; prescreening analysis; herbicide detection

INTRODUCTION

In recent years, world population growth and the consequent increase in food requirements have driven the intensification of foodstuff production at levels not compatible with natural rhythms. In agriculture, this situation has enormously increased the use of pesticides, and in particular of herbicides, contributing to soil and water contamination with obvious repercussions on the environment and human health.

For this reason, each country has issued specific regulations to contain the spread of pesticides and limit consumers' exposure to such potentially dangerous substances. The International Code of Conduct on the Distribution and Use of Pesticides, adopted in 1985 by the Food and Agriculture Organization of the United Nations (FAO) and subsequently updated in 1998 and 2002, establishes noncompulsory standards of conduct for all public and private entities engaged in the distribution and use of pesticides, and since its adoption it has served as the globally accepted standard for pesticide management (1). In Europe, EC Regulation 396/2005 of the European Parliament and of the Council on Maximum Residue Levels (MRLs) regarding pesticides in products of plant and animal origin (entered into force on September 1, 2008) defines a new fully harmonized set of rules for pesticide residues (2). Obviously, the most severe restrictions are those related to food and products intended for children and

young people, whose immature liver enzyme system encounters difficulties in the efficient detoxification of these compounds (3,4). EU Baby Food Directives 2003/13/EC and 2003/14/EC, for instance, fix at a level lower than 10 $\mu\text{g/kg}$ the maximum acceptable daily intake of a series of pesticide residues in cereal-based processed food and processed foods for infants and youngsters.

With regard to herbicides, which represent the most common pollutants found in surface water and groundwater (5), the accepted concentrations are 0.1 $\mu\text{g/L}$ (for each single pollutant) and 0.5 $\mu\text{g/L}$ (for total pesticides) (6). Such a low limit, clearly, requires highly sensitive analytical techniques and reliable methods of analysis. In this context, chromatographic techniques, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC) with UV and/or mass spectrometry (MS) detection, surely represent the most trustworthy techniques for the analysis of complex matrices (7–10). Although these techniques have reached a very high separation power, mainly in multidimensional mode, such as GC $\times$ GC or LC $\times$ LC, by which the peak capacity is increased by performing two independent separations in the same analysis (11, 12), it must be emphasized that pesticides can greatly differ in chemical structure and chromatographic behavior, so it is still impossible to apply a univocal method to discriminate all of the different compounds that could be present in a real-world sample. Depending on the nature of the analyte, indeed, one method can prove more suitable than another, or completely unsuitable. Moreover, pesticides usually represent a very small fraction of the whole sample under

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investigation, so pretreatments such as cleanup and/or preconcentration steps are required to make their identification possible. Taking all of this into consideration, it is evident that laboratories involved in qualitative and quantitative analyses of pesticide residues spend a lot of time preparing and analyzing several samples and applying the proper method for each specific class of pesticide. Because a great number of samples reach these laboratories for routine analyses every day, in general the cost is high and the time required is long.

This work proposes an interesting strategy by means of which a prescreening method based on biosensor technology allows the analyst to considerably reduce the number of samples that must be handled, making the whole procedure easier and cheaper.

According to the IUPAC definition (13), biosensors are devices that use a biological element of recognition, also known as a *biochemical receptor* or *biomediator*, held in direct spatial contact with a transduction system (Figure 1). The biochemical receptors, represented by isolated enzymes, immunosystems, tissues, organelles, or whole cells, translate information from the biochemical domain into an electrical, thermal, or optical output signal, in response to a biochemical variation caused by particular environmental, chemical, or physical conditions. This variation is related to the intensity of the stimulus occurring on the biomediator and can be detected using different detection methods, each having its own advantages and drawbacks.

In the present study, two highly sensitive techniques, such as amperometry and fluorescence emission, were fully explored and exploited to measure the photosynthetic activity variations of

selected biochemical receptors after exposure to samples containing herbicides. Collecting results of independently performed experiments (Table 1), we set out to combine these techniques to obtain more reliable data about the toxicological potential of a given sample containing herbicides. The result of this action led to the implementation of a new technology involving the construction of a platform of biosensors based on photosynthetic biomediators, which offered the possibility of detecting and quantifying different classes of herbicides in an aquatic environment, by measuring the reduction of the biomediator photosynthetic activity as current and/or fluorescence variation.

The advantage of using biosensors is that herbicides can be detected regardless of their nature or the class to which they belong by simply estimating their toxic effect on a living organism. Exploiting prescreening by biosensors, only samples containing critical threshold values would be sent to specialized laboratories for quantitative and reliable GC and/or HPLC analyses, whereas all those that result negative would be discarded.

To extend the use of biosensors to include qualitative analysis, biosensors based on an array of genetically modified algae sensitive to specific classes of herbicides were also investigated, showing their potential at least in the identification of the "class" of chemicals present in a real sample, thereby helping the analyst to properly deal with all further analyses.

MATERIALS AND METHODS

Standard Solutions. Atrazine, prometryn, metribuzin, cyanazine, terbuthylazine, simazine, and deethylterbuthylazine (triazines) and diuron and linuron (ureas) were purchased from Riedel-de Haën (Seelze, Germany). Standard solutions were prepared diluting each herbicide in the proper buffer, according to the biomediator employed and the technique of analysis performed. All chemicals were tested in a range of concentrations between 10^{-5} and 10^{-10} M.

Extraction of Thylakoids. For the extraction of thylakoids, all procedures previously described by the authors were condensed into a unique optimized procedure described as follows. Leaves of *Spinacea oleracea* (purchased in local markets) were homogenized in a medium containing 20 mM tricine, 300 mM sucrose, 5 mM magnesium chloride, 1 mM phenylmethanesulfonyl fluoride (PMSF), and 0.2% (w/v) bovine serum albumin (BSA) adjusted to pH 7.8. The mixture was filtered through two layers of Miracloth and centrifuged for 2 min at 700g. The pellet was discarded, and the supernatant was centrifuged for 10 min at 7500g. The pellet was then resuspended in a rinsing medium containing 70 mM sucrose, 20 mM tricine, and 5 mM magnesium chloride adjusted to pH 7.8 and centrifuged for 10 min at 7500g. The new pellet was resuspended in the

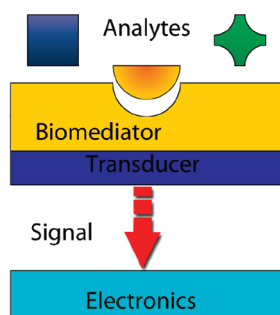


Figure 1. Generic representation of the components of a biosensor system showing the specificity of the biomediator for some particular analytes and the transfer of the signal produced after recognition, from the biomediator to the electronics.

Table 1. Summary of LOD Values and Standard Deviations (SD) Calculated in Our Laboratory for Different Herbicides Using *Chlamydomonas reinhardtii* Cells and Thylakoids from *Spinacea oleracea* as Biomediators for Fluorescence and Amperometric Measurements

biomediator	immobilization procedure	transduction system	herbicide	LOD (M)	SD	ref
<i>C. reinhardtii</i> cells	silicon septum	optical	atrazine	7.3×10^{-10}	1.6×10^{-10}	20
			diuron	2.3×10^{-10}	0.1×10^{-10}	20
			prometryn	3.5×10^{-10}	0.8×10^{-10}	20
thylakoids from <i>S. oleracea</i>	BSA-GA	optical	atrazine	1.6×10^{-8}	0.4×10^{-8}	40
			diuron	1.5×10^{-9}	0.5×10^{-9}	40
			metribuzin	1.0×10^{-8}	0.3×10^{-8}	40
			cyanazine	4.1×10^{-8}	1.1×10^{-8}	40
thylakoids from <i>S. oleracea</i>	BSA-GA on SPE	current	atrazine	1.6×10^{-9}	1.2×10^{-10}	41
			diuron	7.3×10^{-10}	0.7×10^{-10}	41
			terbuthylazine	1.0×10^{-9}	0.5×10^{-10}	41
			linuron	1.2×10^{-9}	0.9×10^{-10}	41
			simazine	2.1×10^{-9}	0.2×10^{-10}	a
			deethylterbuthylazine	5.3×10^{-9}	2.0×10^{-10}	a

^a Unpublished result.

Table 2. Mutants Produced and Relative Sensitivity/Resistance Shown toward Several Herbicides^a

mutant strain	atrazine	diuron	prometryn	alachlor	linuron	terbuthylazine
IL (control)	sensitive	sensitive	sensitive	resistant	sensitive	sensitive
S268C	highly sensitive	resistant	highly sensitive	sensitive	sensitive	sensitive
S264K	resistant	resistant	resistant	resistant	resistant	resistant
F255N	resistant	resistant	sensitive	resistant	nd	nd
A251C	sensitive	highly sensitive	sensitive	resistant	sensitive	sensitive
A250R	sensitive	highly sensitive	sensitive	resistant	sensitive	sensitive
A250L	resistant	highly sensitive	sensitive	resistant	sensitive	sensitive
I163N	sensitive	sensitive	sensitive	resistant	nd	nd
I163Nsd	sensitive	sensitive	sensitive	resistant	nd	nd
L200I	sensitive	sensitive	sensitive	resistant	nd	nd
G207S	sensitive	sensitive	sensitive	resistant	nd	nd
M172L	sensitive	sensitive	sensitive	resistant	nd	nd

^aSensitivity was estimated as $(1 - V_j)$ of the chlorophyll fluorescence curve of each strain at different herbicide concentrations by comparing the results obtained for *C. reinhardtii* wild type. nd, not determined.

rinsing medium and homogenized in the same buffer. All steps were performed at 4 °C in the dark. The amount of thylakoids extracted was quantified by measuring chlorophyll concentration according to the procedure indicated by Arnon (14). The activity of thylakoid membranes was then verified by measuring chlorophyll fluorescence in terms of F_v/F_m (Fluorescence Monitoring System, Hansatech Instruments Ltd., U.K.) and, finally, aliquots were immediately frozen in liquid nitrogen and then stored at −80 °C until used. Alternatively, thylakoids can be lyophilized on specific supports and stored at −20 °C, without any loss of activity for 3 months. In this case, lyophilized adsorbed thylakoids, after regeneration by measurement buffer treatment, can be directly used for fluorescence measurements (as described under Instrumentation), whereas for amperometric use, additional washing and removing steps are required to isolate thylakoids before their immobilization on electrodes.

Preparation of *Chlamydomonas reinhardtii* Wild Types. For the preparation of whole cell biomediators, cell cultures of *C. reinhardtii* were performed in liquid Tris–acetate–phosphate (TAP) at 50 μ mol/m s light intensity, 25 °C temperature, and 150 rpm agitation. Cells were harvested in the midexponential growth phase [up to $(2-3) \times 10^7$ cells/mL], and growth curves were performed at regular intervals. At a specific point of the growth curve, corresponding to a 0.5 optical density (OD) measured by UV at 750 nm, whole cells were collected and utilized at the specific concentration according to the selected immobilization procedure.

Mutagenesis of *C. reinhardtii* Mutants. Mutants were generated by site-directed mutagenesis and PCR as described by Johanningmeier et al. (15) and Preiss et al. (16). The mutagenic primers and the corresponding amino acid change were chosen on the basis of the mutation desired. PCR fragments were amplified from the plasmid pSH5 containing the complete intronless *psbA* gene, encoding the D1 protein and the 3'-flanking regions. The start of the mutation was confirmed by sequence analysis. As reported by Dauvillee et al. (17), the specific PCR-generated *psbA* fragments were directly precipitated onto tungsten particles and inserted into the host by a particle gun. After the transformation, photosynthetically growing colonies were selected and analyzed. In particular, mutants, arising from the results of theoretical calculations, were prepared by site-directed mutagenesis and characterized by electro-optical analysis and used to create an array of biomediators with different degrees of sensitivity and resistance toward chemical species, which are able to discriminate among classes and subclasses of herbicides (triazine, urea, and phenolic compounds). A list of the strains produced is given in Table 2. Strain culture conditions were the same as described for *C. reinhardtii* wild type.

Immobilization Procedures. *Immobilization by Adsorption on Specific Supports.* Immobilization on nitrocellulose membrane filters (Millipore Corp., France) appeared to be a valid method particularly for lyophilized thylakoids. In this case, the filter was used as a support in both the lyophilization and immobilization steps. The procedure was as follows: 0.5 mL of thylakoid extracts at chlorophyll concentration of 3 mg/mL was layered on the filter homogeneously, lyophilized overnight under standard conditions (−50 °C, 0.133 Pa), and stored at −20 °C until used. Immobilization on a silicon septum (Pyrex, Bibby Sterilin Ltd., U.K.), preferred, was carried out by the simple adsorption of fixed amounts of thylakoids resuspended in the proper buffer. The advantage of using porous septa was the possibility of performing dynamic fluorescence measurements in which

the biomediator adsorbed onto the filter could be kept in continuous and homogeneous contact with the buffer or the herbicide solution (18).

BSA–GA Immobilization. All steps were conducted at 4 °C in almost dark conditions, slightly modifying the procedure reported by Koblizek et al. (19). A 20% w/v BSA solution was added to a sodium phosphate buffer solution (50 mM, pH 7.1) and mixed until the protein was fully dissolved. A 1.5% w/v GA solution was added, and the mixture was incubated for 2 min. Thylakoid suspension was then added, and the final mixture was mixed for 5 s (by vortex mixer) and distributed onto the electrode surface by means of a micropipet (5 μ L for each graphite working electrode). The electrodes with immobilized thylakoids were kept at −80 °C for 12 h minimum.

Instrumentation. The core of the constructed platform was composed of two different devices: the OPTICBIO-Multicell fluorometer and the AMPBIO-SPE amperometer (Biosensor S.r.l., Rome, Italy, www.biosensor.it). The first biosensor was equipped with 96 LEDs with different emission wavelengths for fluorescence excitation and 24 silicon photodiodes and optical filters for fluorescence emission measurement. Each photodiode provided a spectral response in the range of 300–1100 nm, and different band-pass optical filters were used to select the set fluorescence peak: in the case of *C. reinhardtii* organisms, a bandwidth centered at 720 ± 5.0 nm was selected. The instrument automatically calculated the fluorescence parameter $(1 - V_j)$. It was equipped with 24 measurement cells (4 arrays of 6 cells each, 10 mm external diameter, 12 mm height), arranged in two physically and functionally distinct compartments, separated by a polycarbonate window (see Figure 2A). The optical module at the bottom was mounted inside the instrument case and provides fluorescence excitation and detection. The biological module, perfectly sealed, hosted biomediators immobilized on porous septa (destined to fluidic measurements) or suspended biomediators (destined to static analyses): slightly different cell arrays that could be easily and quickly exchanged if necessary were used in these two cases. Both static and dynamic operations were carried out using an automatically controlled fluidic system. Repeated fluorescence measurements were automatically performed by diluting samples in a buffer able to maintain the vitality of the cells. LODs, determined for *C. reinhardtii* wild type and mutants according to the method of Koblizek et al. (19), have recently been reported (20).

The second instrument, used for amperometric measurements, was based on a special unit where biological material, electrode, excitation light source, and flow were integrated in a compact, miniaturized sensor cell (30 mm external diameter, 25 mm height). As shown in Figure 2B, the biological chamber where the biomediator was placed and the reaction with the target analyte occurred was made up by the top overlap of the screen-printed electrode (SPE) and the O-ring. The internal volume (defined by the thickness of the O-ring, which equaled 22 μ L) was so small that the flow exchanged very quickly and the kinetic of the biochemical reactions was improved, thus shortening the response time. The biomediator was placed on the SPE and held in place by a screw-type adjustment system made up of a piston and a screwed tap, which pushed the SPE against the O-ring to expose the biomediator to the solution containing buffer and herbicide. A glass window separated the biological chamber from the optical compartment. The electrolytic solution flowed

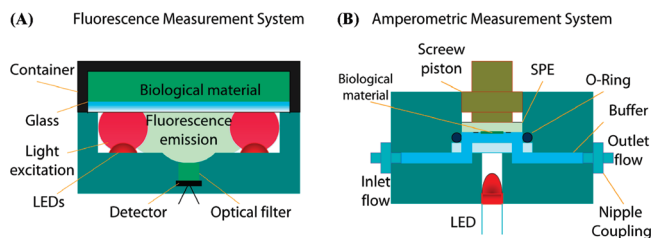


Figure 2. Schematic cross section of fluorescence (A) and amperometric (B) measurement cells, both made up by two independent compartments for biological material (on top) and transduction elements (at the bottom).

into the chamber through two small holes, gently filling the volume and preserving the biomaterial from physical damage or detachment from the electrode. The optical module, placed on the bottom, induced the biosample excitation and sustained the subsequent light-derived electron transfer process. Two LEDs with emission wavelengths of 470 and 650 nm were used to provide different excitation sources for different biomediators and target the maximum photosynthesis rates. A LED timer function allowed the modulation and setting of the excitation light interval, thus controlling the photoperiod with the precision of 1 μ s. The electrolyte solution was pushed into the measuring cells by a rotary peristaltic pump with adjustable speed (200–350 μ L/min). The fluidic system was fully automatic and controlled by a timer that allowed the pump flow and time interval to be set.

Measurement Procedures. *Fluorescence Measurements.* Fluorescence measurements were performed in static and dynamic mode using TAP buffer at pH 7.0. For dynamic measurements, *C. reinhardtii* cells (wild type and strains) were previously immobilized on silicon septa. Calibration standards were prepared by diluting herbicides in the TAP buffer. All samples and standard solutions were analyzed in triplicate, after 10 min of conditioning in the dark, flashing the sample for 5 s (by red LED at 650 nm, 1500 mCd) to saturate the system and measuring the 1 s response obtained after switching off.

Amperometric Measurements. Amperometric measurements were performed in static and dynamic modes on thylakoids from *S. oleracea* after their immobilization on graphite screen-printed electrodes. The measuring buffer was a solution of tricine (20 mM), $MgCl_2$ (5 mM), sucrose (70 mM), and NaCl (50 mM) at pH 7.2. Different mediators had been added to the buffer in the past; in this reported case, only 2,6-dichlorophenolindophenol (DCPIP, 30 μ M) was employed. All samples and standard solutions were analyzed in triplicate in the same conditions employed for fluorescence measurements (10 min conditioning in the dark, flash for 5 s by red LED at 650 nm, 1500 mCd) and applying a potential of 0.200 V with a current of 100 μ A.

RESULTS AND DISCUSSION

Choice of Biomediator. Biosensors are extremely selective devices; their features are strictly related to the nature of the analytes to be detected. In the present study, it was considered a specific class of biosensors based on the use of photosynthetic biomediators conceived for the detection of different groups of herbicides. In recent years, different organisms have been employed as photosynthetic biomediators (21, 22); among these, *S. oleracea* and, more recently, *C. reinhardtii* (23) have been successfully exploited. The major factor responsible for their photosynthetic activity is represented by photosystem II (PSII), an enzymatic chlorophyll–protein complex that is the real sensing element of PSII-based biosensors. The advantage of using these biomediators is that PSII recognizes many environmentally important classes of herbicides, such as triazines, phenylurea, diazines, and phenolic compounds, which today are still widely employed for weed control in agriculture.

Located in the thylakoid membrane of algae, cyanobacteria, and higher plants, PSII is a light-driven, water–plastoquinone oxidoreductase, which represents an integral part of the electron transport chain implicated in photosynthetic primary charge

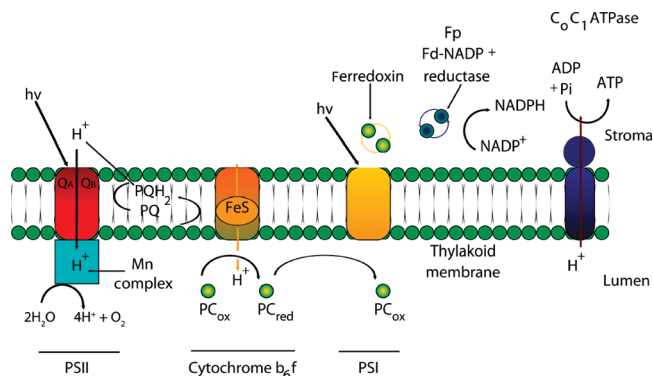


Figure 3. Model of the photosynthetic membrane of plants showing the electron transport components (cross-sectional view). The complete membrane forms a vesicle. The pathways of electrons are shown by solid arrows. Photosystem II (PSII), photosystem I (PSI), and cytochrome b_6f complexes, all involved in the electron transfer chain, are also shown.

separation. The electron flow begins with the release of an electron from the excited P680 molecule to the primary pheophytin acceptor. This charge separation is stabilized by the transfer of an electron to quinone Q_A , a firmly bound plastoquinone located in the D2 subunit of PSII, and subsequently to Q_B , a mobile plastoquinone located within the D1 subunit of PSII. Q_B is a two-electron carrier, and whereas the partially reduced Q_B^- form is tightly bound to the D1 protein (called the Q_B pocket), the fully reduced and protonated Q_BH_2 form has a relatively low affinity for the hydrophobic pocket of D1. Consequently, another molecule of the plastoquinone pool displaces Q_BH_2 , occupying the D1 binding pocket (24), and a new electron transfer can take place (Figure 3). Most herbicides act by displacing the plastoquinone from the Q_B site of the D1 protein, thus interrupting the electron flow and oxygen evolution, changing directly the fluorescence properties of PSII (21).

For the preparation of PSII-based biomediators, both whole photosynthetic cells and isolated organelles can be used. In general, 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 toward 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.

In our studies, whole photosynthetic cells from *C. reinhardtii* and thylakoids from *S. oleracea* were taken into consideration. For fluorescence experiments, both cells (25) and organelles gave good results, demonstrating that the choice of using whole cells or subparticles is simply driven by a matter of choosing between lower efficiency biomediator systems and longer extraction procedures. Early amperometric measurements, on the contrary, were successfully performed only on thylakoid extracts, because the transmission of the electrical signals through the thick external layer of *Chlamydomonas* cell wall was irregular and not always reproducible. Only recently, a new procedure still under investigation (data not shown) has demonstrated the possibility of using *Chlamydomonas* cells for both optical and amperometric measurements, but further improvements of the instrumentation are needed to raise the sensitivity and signal-to-noise ratio.

Immobilization of Biomediators. After isolation, the activity of cells and photosynthetic materials undergoes a rapid decrease. To preserve their vitality during fluorescence and amperometric

Table 3. Immobilization Methods Applied to Thylakoids Extracted from *S. oleracea*^a

physical immobilization method	RA ^b	leaking	chemical immobilization method	RA	leaking
adsorption			reticulation		
filter paper disk	56	high	glutaraldehyde	70	low
alumina filter disk	26	high	magnetic beads polymer	60	low
glass microfiber filter	49	high	binding on gold films	35	low
DEAE cellulose	50	high			
nitrocellulose	90	medium			
gel inclusion			co-reticulation		
polysaccharide gel			glutaraldehyde (different proteins)		
agar	45	medium	gelatin	42	low
agarose	33	medium	collagen	30	low
carrageenan	20	low	bovine serum albumin	70	low
alginate	20	low			
protein gel			<i>cross-linking on inorganics</i>		
gelatin	50	low	gold	20	low
			TiO ₂	55	low
Langmuir–Blodgett	50	high	<i>synthetic gel and polymers</i>		
lyophilization	60–80	high	polyacrylamide	41	low
			polyurethane	28	low
			photo-cross-linkable resin	10	low
			vinyl	15	low
			poly(vinyl alcohol)	38	low
			tyrpyridinium groups	45	low
			thiophen polymer	57	low

^a The stability of each procedure was evaluated measuring the *leaking* of the material and its *residual activity* (RA) before and after immobilization. ^b Residual activity after immobilization (measured as percentage of the initial value in terms of oxygen evolution).

measurements, 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 unfortunately 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 past 10 years (19–25). 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 has already been demonstrated (26), this cross-linker, which does not affect the Hill reaction activity that leads to oxygen production, allows the immobilization of the photosynthetic material (27) with a slight denaturing effect, provided proteins are added during the phase of polymerization.

In this study, our aim was to identify generally suitable procedures that could be applied to amperometric and/or fluorescence measurements. **Table 3** summarizes all of the immobilization methods tested, so that the most appropriate in terms of stability can be selected. Each method was tested on thylakoids extracted from *S. oleracea*, assuming that results obtained for thylakoids can also be considered valid for *C. reinhardtii*.

Physical Methods. Among the physical methods tested, adsorption and gel inclusion appeared to be the most suitable. Various sorbents were investigated as support materials: filter paper disks, alumina filter disks, glass microfiber filters, and columns containing diethylaminoethyl-cellulose (DEAE-cellulose). The immobilization was obtained by simple filtration or by lyophilization on the support. In many experiments, after adsorption, the filter containing the biological material was protected with a thin

alginate layer hardened with CaCl₂ or with another filter disk, giving rise to a physical–chemical method of immobilization. With regard to the gel inclusion methods, natural or synthetic gels were employed. Among the former, polysaccharides, agar, agarose, carrageenan, and alginate were tested, whereas gels of polyacrylamide, polyurethane prepolymer (PU-3), photo-cross-linkable resin prepolymer (ENT-4000), vinyl monomers (HEA or M-23G), poly(vinyl alcohol) polymers, and poly(vinyl alcohol) bearing styrylpyridinium polymers (PVA–SbQ) were used as synthetic gels. The polymerization of some synthetic gels, required to make the entrapment, was initiated by specific chemicals (polyacrylamide gel) or by irradiation (UV light was used for PVA–SbQ, white light for photo-cross-linkable resin prepolymer, γ -rays for vinyl monomers). Another interesting physical method of immobilization, not reported in the table, is that performed by Nafion (perfluorinated resin, 10 wt % aqueous dispersion), a very popular polymer with ionic properties that has been employed since 1996 for enzyme entrapment (28) and is being investigated by us for use in thylakoid and cell immobilization on electrodes.

Chemical Methods. Among chemical methods, the immobilization by bovine serum albumin–glutaraldehyde (BSA–GA) mixtures was selected for most of the amperometric and fluorescence measurements performed (see **Table 1**). The advantage of coupling GA with BSA is that the network of covalent bonds built by GA involves the free –NH₂ groups of both the photosynthetic material and the exogenous protein, thus reducing the denaturing effect on the biomediator.

Two different immobilization procedures, based on the results obtained, were selected as the most suitable: immobilization on specific supports, such as nitrocellulose filter and silicon, and BSA–GA immobilization. The former was applied to perform only fluorescence measurements, whereas the latter was applied to both fluorescence and amperometry.

The stability of both the physical and chemical procedures was evaluated by measuring the leak of material and its photosynthetic activity before and after immobilization. To estimate the extent of the leak, fixed volumes of buffer were pushed through the flow cell containing the entrapped biomediator at a constant rate. The solution from the cell was then analyzed by the UV

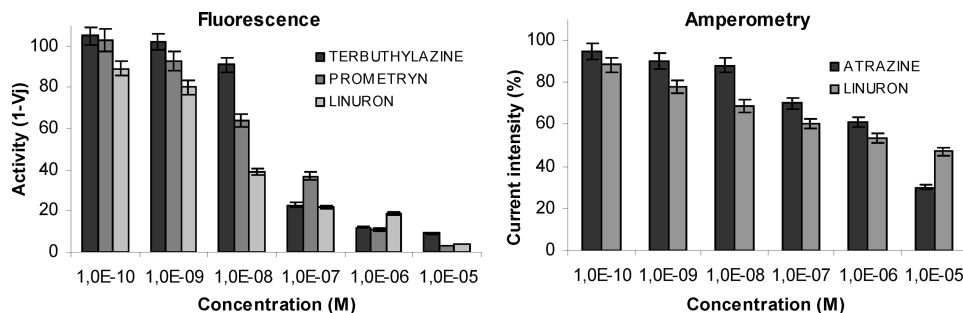


Figure 4. Diagrams of calibration showing different herbicides analyzed by optical (left) and amperometric biosensors (right) by using *C. reinhardtii* and spinach thylakoids, respectively.

spectrophotometer to reveal if there were traces of chlorophyll (absorbance at 652 nm), proving the presence of released photo-synthetic material. As shown in the table, three levels of leaking were defined (low, medium, and high), and the lowest levels were obtained by applying chemical methods, as reticulation and coreticulation. To be sure to determine if the selected immobilization procedures were also able to preserve the vitality of thylakoid membranes, their photosynthetic activity was measured before and after immobilization in terms of oxygen evolution (Oxylab, Hansatech Instruments).

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 in converting the biochemical signal into an electric signal. 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. This is why we focused our attention on these two techniques, developing two new biosensors able to detect, by performing parallel fluorescence and amperometric measurements, the signals produced by the biomediator in response to light excitations of a specific wavelength.

Diagrams of calibration were constructed for each herbicide both on the optical and on the amperometric biosensors. **Figure 4** illustrates two examples of diagrams showing different herbicides analyzed either by fluorescence or by amperometry.

Fluorescence. Chlorophyll fluorescence is a sensitive, noninvasive, 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 (29): actually, most of the light energy is involved in photosynthesis, whereas 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.

The presence of herbicides, due to the high affinity of these compounds toward the Q_B site of the D1 protein of PSII (see **Figure 3**), 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, increasing its yield, which can be easily measured and which corresponds to the concentration of herbicide.

Fluorescence measurements reported in the present study refer to the fluorescence induced by artificially illuminating the

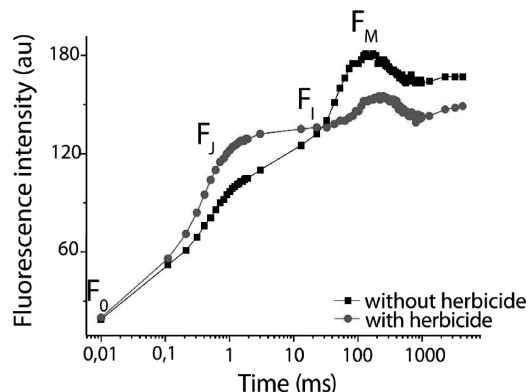


Figure 5. Typical Kautsky fluorescence transient exhibited by dark-adapted *C. reinhardtii* whole cells immobilized on silicon septa, upon illumination by saturating light at 650 nm wavelength. F_0 , F_J , F_I , and F_M parameters are shown before (black line) and after (gray line) the addition of herbicide. Buffer, Tris–acetate–phosphate (TAP), pH 7.0; dark time, 10 min; light time, 5 s (by red LED at 650 nm, 1500 mCd); instrument, OPTICBIO-Multicell fluorometer.

biomediators. Fluorescence rises quickly upon illumination from an initial fluorescence intensity, F_0 , to a maximal intensity, F_M , reached after about 300 ms. Between these two extremes the fluorescence intensity shows intermediate steps, such as F_J (measured at about 2 ms) and F_I , which describe a typical Kautsky fluorescence transient curve (**Figure 5**). Due to the typical shape of the fluorescence curve, which shows the steps F_0 , F_J , F_I , and F_M , information about the activity of the electron transport beyond Q_A^- and the photosynthetic metabolism can be easily deduced. In particular, the relative variable fluorescence $V_J = (F_J - F_0) / (F_M - F_0)$, and consequently the parameter $(1 - V_J)$, both depending on the redox state Q_A^- / Q_A of the sample in a given physiological state, can be calculated. This procedure simplifies the analysis by considering only the dynamic accumulation of Q_A in its reduced form, Q_A^- (30). Fluorescence measurements consist in determining the variation of one of these parameters, in response to environmental or chemical changes.

Amperometry. Electrochemical techniques, such as amperometry, exploit the fast and direct electron transfer activated by light energy to transduce it externally into an output current. In this case, the current signal is measured between the working electrode featuring the immobilized biomediator and the so-called counter electrode, and, if required, it can be successively amplified for easier processing and data analysis. A typical amperometric measurement, which refers to plant thylakoids, is shown in **Figure 6**. To technically evaluate the photosynthetic activity of PSII, an artificial electron acceptor (also called a mediator) is solubilized in the buffer used for all measurements.

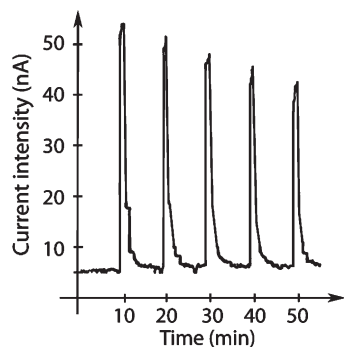


Figure 6. Amperometric measurement performed in static mode on thylakoids from *S. oleracea* immobilized with BSA-GA on a screen-printed electrode. Atrazine is added at intervals of 10 min at four different concentration (10^{-9} , 5×10^{-9} , 10^{-8} , and 5×10^{-8} M) starting from 20 min. Buffer (pH 7.2), tricine (20 mM), MgCl_2 (5 mM), sucrose (70 mM), NaCl (50 mM); mediator, DCPIP (30 μM); dark time, 10 min; light time, 5 s (by red LED at 650 nm, 1500 mCd); instrument, AMPBIO-SPE SS LIGHT; potential, + 0.200 V; current, 100 μA .

Once the system is illuminated (at minute 10 in the graph), the photosynthetic reaction takes place, reducing the electron acceptor; at that point, if a suitable potential (+200 mV for thylakoids) is applied between working and reference, the acceptor releases its charge at the electrode surface and quickly goes back to the oxidized form. As a consequence, a current peak proportional to the photosynthetic activity can be recorded and used as blank signal. In the presence of herbicides, the Q_B site of the D1 protein is blocked and the current output normally recorded in response to light excitation decreases proportionally with the pollutant concentration. **Figure 6** shows the reduction observed in terms of current intensity when atrazine at concentration of 10^{-9} , 5×10^{-9} , 10^{-8} , or 5×10^{-8} M is added to the measuring buffer.

Molecular Biology for Qualitative Analyses. The PSII-based biomediators so far described can detect all classes of photosynthetic herbicides inhibiting the Q_B site of the D1 protein, such as diazines, triazines, phenylureas, and phenolic compounds, but cannot give any information about the specific class or compound present in a sample solution. To obtain qualitative analyses, our efforts were directed to the generation of genetically modified strains of *C. reinhardtii*, in which the affinity of the Q_B site of the D1 protein to herbicides was modified. Depending on the modification that occurs, algae may be more or less responsive to a given compound. This means that by cross-examining properly the information from different strains, the analyst can predict which herbicide is present in the sample analyzed. Early experiments on tap water samples, previously spiked with known amounts of standard herbicides, confirmed that the selected mutants can predict the presence of specific compounds. Real-world matrices such as fruit juice, homogenized vegetables, and olive oil are currently being investigated.

***C. reinhardtii* Strains and Array of Mutants.** The unicellular green alga *C. reinhardtii* was one of the first model organisms in cell biology. The advantage of this organism, for which the first studies date back to 1940, is that it is easy to grow and it lends itself to genetic and molecular manipulations, allowing it to be applied to many fields (31, 32).

To set up an array of mutants useful for our aims, computational, genetic, and bioinformatics studies were carried out (33, 34). In particular, we focused our attention on functional amino acids involved in the interaction of the D1 protein with herbicides. Notably, the herbicide-binding site consists of about 65 amino acids, and even only one modification on one amino

acid is enough to considerably change photosynthetic activity and herbicide binding. Depending on the position and type of amino acid substitution, the affinity of the D1 binding niche for chemically different inhibitors can greatly change and, surprisingly, a mutation that causes resistance toward one class of inhibitors sometimes results in hypersensitivity toward another class (35).

In our studies, a series of D1 protein mutants were generated in silico (see **Table 2**), to construct an array of mutants based on the different affinity of each strain to a specific class of herbicides. Each mutant was tested with different triazinic and ureic herbicides in a range of concentrations between 10^{-5} and 10^{-10} M. Taking into account the library of responses of each mutant to the single herbicide in question, it was possible to determine the presence/absence of that herbicide in a solution by simply evaluating its effect on the fluorescence properties of the mutant. **Figure 7** shows the different behavior of two mutants when different herbicides are present. Thanks to their high specificity, the operator can predict which herbicides (or class of herbicides) are present in a sample by simply evaluating the inhibition produced on the single organisms used as biomediators.

Biosensor Platform. The platform described in this paper consists of two new instruments conceived for parallel fluorescence and amperometric measurements in static or dynamic mode. The platform was constructed on the basis of results collected over many years of experience. In particular, starting from the most significant experiments reported in **Table 1** and taking into account results described in **Table 3**, a protocol of analysis was drawn up, whereby the operator can quantify the total amount of herbicides in a solution and identify their class. **Figure 8** is a scheme describing the fundamental steps of the protocol, before HPLC or GC intervention. Briefly, after sample preparation (see Materials and Methods), fluorescence and amperometric tests can be performed by pushing known volumes of the sample previously diluted in the proper measuring buffer through the flow cells of each instrument and measuring any inhibition signals that occur on the biomediator after contact with the sample solution. Dedicated software allows the operator to read-out these signals, quantifying the analytes and identifying their class. The identification is possible by carrying out fluorescence experiments on multicell instruments, instead of single ones: in this way, each mutant is located in a different cell, and depending on the responses from the totality of the cells, the class can be predicted. Measurements consist in registering and automatically comparing the photosynthetic activity in normal conditions and in the presence of herbicides to estimate the quantitative effect of a given herbicide by correlating its concentration with the inhibition induced on the biological material. The sensitivity of the living organisms used as biomediators is so high that the operator can immediately define the risk level of the sample analyzed, sending suspected samples to HPLC or GC laboratories for more detailed analyses.

As demonstrated by our experiments, fluorescence and amperometry allowed the quantification of the herbicide residues in a wide range of concentrations; however, when experiments were carried out separately, some serious discrepancies were observed among the results obtained. The reason for these discrepancies is that herbicide-induced damage can involve different regions of the PSII complex; for instance, any damage involving the manganese complex of the oxygen evolving complex (known as OEC) leaves the fluorescence properties unaltered, so they cannot be revealed by fluorescence measurements, consequently giving false negatives. In this case, amperometry acts as an important supporting technique, because the inhibition of water oxidation related to the damage on manganese complex does not affect fluorescence but blocks the PSII electron transfer, reducing the

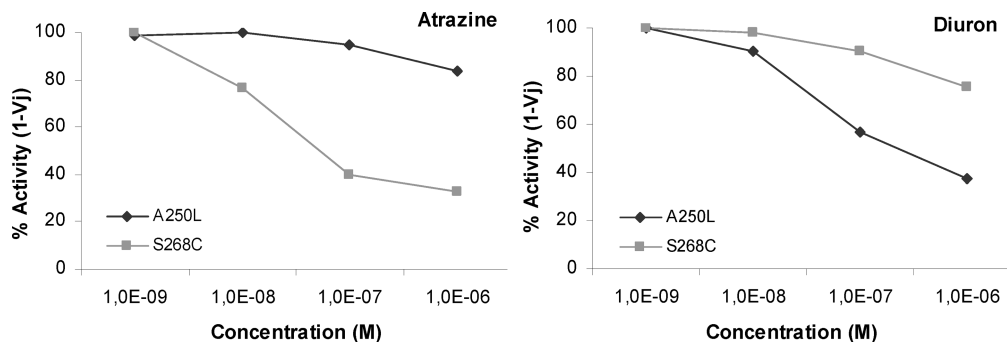


Figure 7. Diagrams showing the different behaviors of two mutants (A250L and S268C) in the presence of increasing concentrations of atrazine and diuron. By comparing the set of responses of many mutants, it is possible to predict which herbicide is contained in a sample by simply evaluating the percentage of inhibition produced on algae.

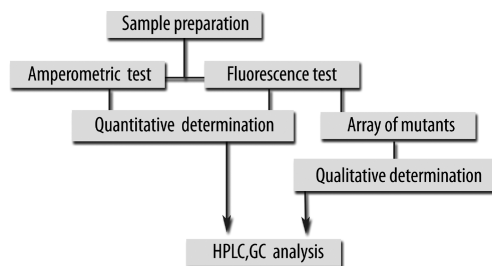


Figure 8. Flowchart showing the phases of analysis performed by using the platform described. Employing spinach thylakoids or *C. reinhardtii* wild types, amperometric and fluorescence tests allow quantitative analyses that can be supplemented with qualitative information by measuring fluorescence on arrays of *C. reinhardtii* mutants able to identify specific classes of herbicides.

output current signal detected (36). In general, from our experiments, we observed that amperometric measurements reach lower limits of detection (LODs) than fluorescence. For instance, by comparison of results obtained on thylakoids from *S. oleracea*, immobilized with BSA–GA (see **Table 1**), it is possible to see how LODs calculated by amperometry, in the cases of both atrazine and diuron, were 1000-fold lower than those calculated by fluorescence. On the other hand, amperometry can be affected by several redox compounds present in the natural samples, whereas fluorescence is more specific to certain classes of compounds than amperometry. Taking all this into account, it can be said that coupling the two techniques in one platform can give additional and/or complementary information about the state of the photosynthetic apparatus, allowing the analyst to get fewer unambiguous results than those obtained by applying only one of the two techniques.

Possible Applications. Judging from results previously obtained by authors on water samples (37, 38), the described biosensor platform can be considered a useful tool for the prescreening of river and spring water. The only disadvantage, for such kind of analysis, is the slight PSII overactivation already observed by Touloupakis et al. (37) when performing amperometric measurements. However, this phenomenon, partly due to the high concentration of divalent cations in river water and in accordance with the knowledge that PSII activity depends on the presence of charge ionic species (39), can be easily recognized (and quantified) by using the internal standard method. Our next step forward, probably with a significant impact in biosensor technology, will be the analysis of food matrices, such as freshly prepared fruit juice, homogenized vegetables, and olive oil. Obviously, the complexity of these matrices is higher than that of water, thus requiring longer extraction and purification procedures.

In conclusion, we can affirm that the biosensor platform we have developed and described represents a sensitive, reliable, and low-cost system able to detect water pollutants such as atrazine, diuron, linuron, and terbuthylazine down to a concentration range of 10^{-10} – 10^{-8} M. Combining the amperometric and optical responses from the platform, the analyst can determine the toxicological potential of samples by estimating the effect produced on the organism used as a biomediator in terms of inhibition of its physiological activity. Fluorescence increase and current reduction are related to the concentration of herbicide present in the sample and can be easily quantified by dedicated data acquisition software able to process data and plot the results.

The novelty of the platform in question is that by using different strains of genetically modified algae, the class of herbicides can be identified, allowing both quantitative and qualitative sample analysis to be carried out. Moreover, the whole instrumentation has been designed to be easily used, so that no highly skilled workers are needed and, due to the use of water as single measuring solvent, the impact of the technique on the environment is practically negligible. Despite all of these advantages, the described PSII-based biosensor platform is not intended for use in detecting herbicide concentrations lower than 10^{-10} M. As far as these are concerned, HPLC and GC (mainly if coupled to MS) are still considered to be the techniques of choice. However, for routine analysis, when hundreds of samples must be daily screened, the biosensor platform represents a useful, easy, and fast way to drastically reduce the number of tests. By performing an in-field prescreening of samples, those that result “negative” can be discarded and only the positive ones considered for further laboratory investigation.

ABBREVIATIONS USED

BSA, bovine serum albumin; GA, glutaraldehyde; OD, optical density; PSII, photosystem II; LODs, limits of detection; SPE, screen-printed electrode.

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