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A new miniaturized multiarray biosensor system for fluorescence detection

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Abstract

A miniaturized biosensor-based optical instrument has been designed and fabricated for multiarray fluorescence measurements of several biomediators in series, with applications in environmental monitoring and agrofood analysis. It is a multicell system featuring two arrays of five static cells ($1 \times 1 \times 2$ cm^3) which are sealed to avoid contamination. Every cell is made up by two modular sections: the bottom compartment with optical LED light excitations and a photodiode detector for fluorescence emission capture, and the top biocompatible compartment where the biosample is deposited. The system (0.250 kg without batteries and case, $100 \times 100 \times 150$ mm^3 internal case dimensions) is equipped with electronic control boards, a flash memory card for automatic data storage, and internal batteries, thus being portable and versatile. The instrument allows one to perform simultaneous and multiparametric analyses and offers a large applicability in biosensor technology. The first prototype has been implemented with genetically modified oxygenic photosynthetic algae that were employed in the instrument experimental testing by monitoring pesticide pollution in water. Pesticides modify the photosystem II (PSII) activity in terms of fluorescence quenching. The PSII complex features a natural nanostructure and can be considered a sophisticated molecular device. Results from measurements employing several PSII mutants and six different pesticides at increasing concentrations and incubation times are presented and discussed.

(Some figures in this article are in colour only in the electronic version)

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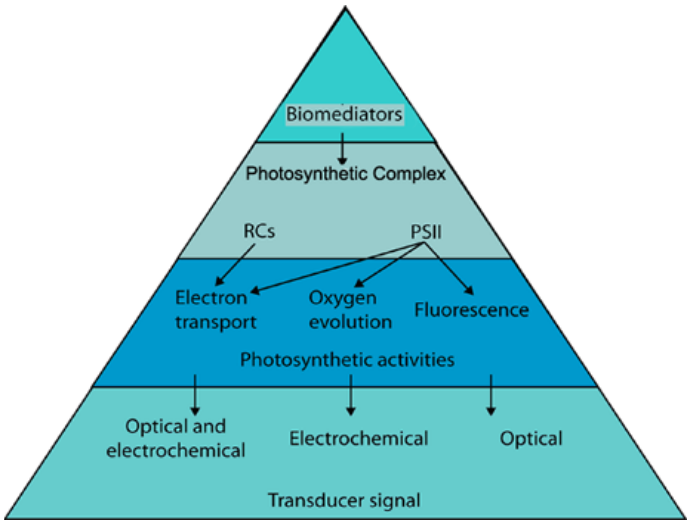

Figure 1. Representation of the sub-components of a biosensor system employing a photosynthetic biomediator.

1. Introduction

A biosensor links a biologically active compound, called the biomediator, to a transducer able to give an electrical or optical measure of the biomediator activity variation in response to particular environmental, chemical or physical conditions. Figure 1 schematically represents the concept of a biosensor based on a photosynthetic biomediator, whose interactive component can be either the reaction centre (RC) or the photosystem II (PSII), natural nanostructured complexes which behave as sophisticated molecular devices [1]. Three different photosynthetic activities can be monitored to detect the chemical/physical interactions: electron transport, oxygen evolution and fluorescence emission. According to the observed phenomenon, different detection mechanisms and transducers can be implemented: electrochemical, amperometric and optical.

One of the most commonly employed methods is the optical one, in which the emitted fluorescence signal, previously excited by a proper light source, is measured. Fluorescence can be an intrinsic property of the biological material, like in the case of a photosynthetic complex, or otherwise induced by using specific fluorescent probes (e.g. DNA, proteins).

Several techniques and measurement systems have been widely employed in the field of plant physiology to monitor photosynthetic activity: e.g. a Fraunhofer line discriminator coupled to a low-pressure cell containing oxygen and a photomultiplier tube [2]; a filtered CCD camera detector that captures a complete image of the sample [3] (e.g. FluorImager, Molecular Dynamics Inc.); a laser-induced fluorescence spectroscopy coupled to CCD imaging [4], especially in remote sensing applications [5]. In 1931, Kautsky and Hirsch [6] described a typical variation of a plant fluorescence spectrum in response to light exposure, subsequently named the Kautsky effect (also called fluorescence transient, fluorescence induction or fluorescence decay). A bench apparatus for herbicide detection has been previously reported by some of this paper's authors: this instrument allowed chlorophyll fluorescence measurement and Kautsky emission detection of photosynthetic algae immobilized in a single cell, by using a modified commercial plant efficiency analyser from Hansatech Ltd (EU Patent 2006 [7]) [8].

All the above-mentioned set-ups are not suitable for portable applications (size, weight, integrability) and do not represent cheap solutions in terms of the choice of source and detector devices. The instrument described in this paper is a compact autonomous integrated light-weight system, with innovative improvements with respect to other commercial fluorometers (e.g. Opto-Sciences http://www.optisci.com/apf.htm) as follows: (1) the modularity of the measurement cell containing the biological material, which allows easy maintenance and parts replacement and the biosample change without disposing or modifying the optical head; (2) the contemporary real-time fluorescence measurement of several biosamples in series due to the multicell configuration and the synchronization mechanism of the analysis (Patent registered in July 2006 [9]); (3) the possibility to perform physiological multiparametric analyses due to different LED light sources able to match the huge number of commercial fluorophores (Patent registered in December 2006 [10]); (4) the miniaturized size in relation to its performance.

2. Photosynthetic biomediators

In this paper, experimental results obtained by using photosynthetic biomediators are described. In addition, other chemical compounds featuring fluorescence emission capability and/or fluorescent probes can be employed and measured.

Photosystem II, represented in the electron transfer chain in figure 2(a), is the multi-enzymatic chlorophyll–protein complex located in the thylakoid membrane of algae, cyanobacteria and higher plants. It is an integral part of the electron transport chain that catalyses photosynthetic primary charge separation. This protein complex consists of over 25 polypeptides, which make up the light-harvesting chlorophyll protein complex, the reaction centre and the water-splitting system, also called the oxygen evolving complex (OEC).

The PSII complex also contains the target site of the most widely used photosynthetic pesticides. Under illumination, PSII drives electron transfer which is inhibited by specific chemical compounds. Therefore, the photosynthetic membrane isolated from higher plants and the whole photosynthetic micro-organisms, immobilized and stabilized, can serve as the biosensor biomediator. The effect of compounds that alter or inhibit photosynthetic activity, measured as fluorescence emission variation, can then be translated and monitored by optical systems. The biosensor system is specific to photosynthetic inhibitors (represented by triazines, ureas, diazines, phenols) that can bind to proteins of the PSII complex. Phenylurea, triazine and diazine, heavy metals and phenolic compounds represent economically important compounds and are widely used in chemical, pharmaceutical and agricultural industries. These compounds have been synthesized since the Second World War and have been applied in great quantities, particularly as herbicides and explosives.

Figure 2(b) shows the so-called photochemical quenching in response to two different excitation light intensities and the administration of a photosynthesis inhibitor, which strongly affect the Kautsky curve. In the last sections of this paper experimental measurements performed with different inhibitors at different concentrations are reported and discussed.

3. Biosensor system design and fabrication

The multicell fluorescence detector has been designed and fabricated in collaboration with Carso (Center for Optical Space Systems, Trieste, Italy). It is made up by two arrays of five cells each (figure 3) allowing for ten contemporary analyses thanks to a synchronization mechanism. Modularity and configurability are implemented through the separation of each cell into two independent sub-modules (figure 4), a fixed bottom section lodging the optical devices and a

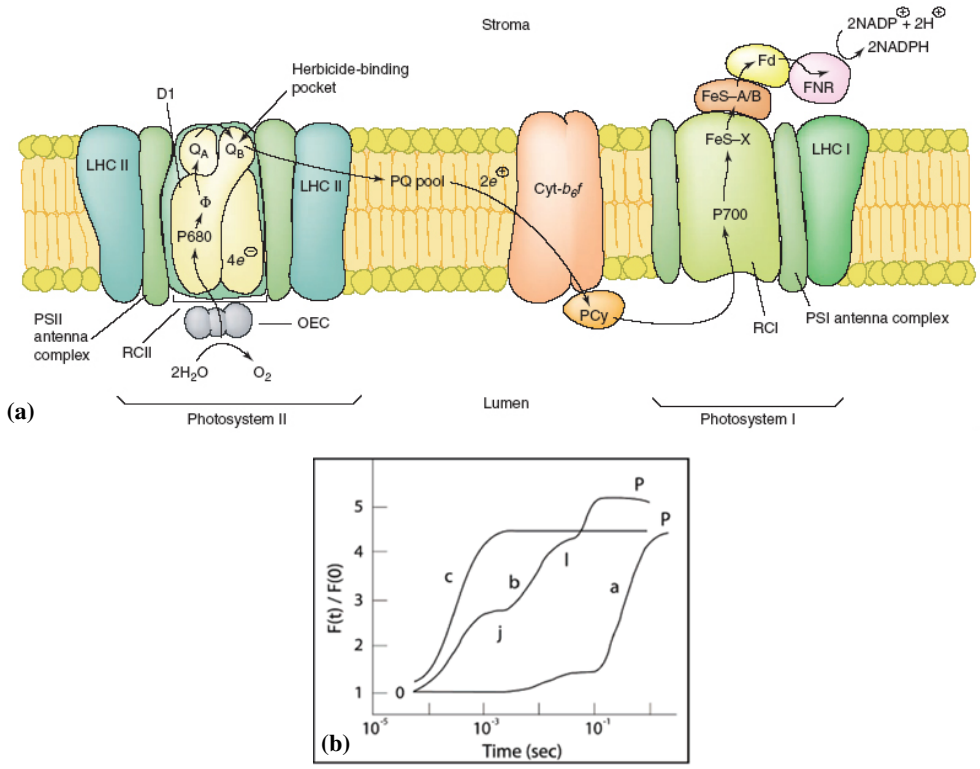

Figure 2. (a) A schematic representation of the electron-transfer chain occurring in the reaction centre during photosynthesis; the pesticide binding site in the Q_B quinone pocket of the D1 protein of PSII is also indicated; (b) fast chlorophyll *a* fluorescence induction curve versus time measured on dark-adapted spinach illuminated at 650 nm with 70 μmol m^{-2} s^{-1} intensity (graph a), 300 μmol m^{-2} s^{-1} intensity (graph b) and in the presence of herbicide diuron (graph c). Figure adapted from [6].

removable upper section containing the biosample, that can be easily disposed after testing and replaced with different biomediators for new analyses.

Four excitation LED light sources with the desired output wavelength and a fluorescence emission photodiode detector can be mounted in each cell. Figure 5 represents the field of view (FOV) of one of the LED sources in the cell.

Each photodiode is coupled to a different interferential band pass filter, tuned to the desired fluorescence intensity peak. The photogenerated output current is amplified and converted into a voltage signal by a read-out electronic circuit, which is coupled to an electronic control board driving the LEDs on/off according to a programmed timer. For the experiments described in section 4 two red LEDs (650 nm) have been used to match the exact excitation wavelength of the photosynthetic biomediators. Moreover, in this specific application each biosample is kept alive by two additional survival white LEDs, mounted close to the exciting ones, in order to enable long-term measurement campaigns implementing day/night cycles with a controlled timer. All LED light intensities can be controlled and set electronically.

Measurement data are stored onboard, through a 12-bit resolution sampling at 480 kHz and ADC conversion, in a flash memory card to retrieve the information at any time with maximum flexibility. A solid-state temperature sensor is also provided in order to measure and store

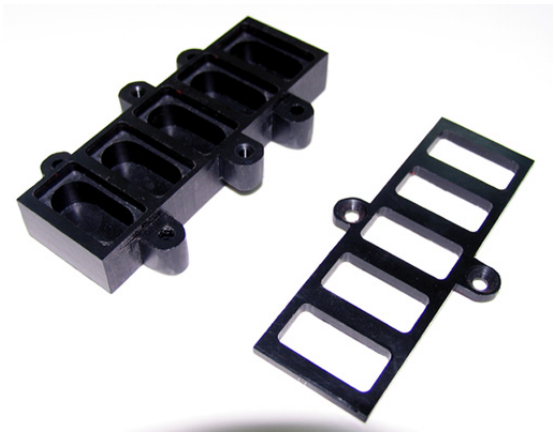

Figure 3. Array of five containers for the biological material. Once sealed with transparent polycarbonate windows, the module is attached to the optical section, steadily mounted on top of the electronic board (figure 6).

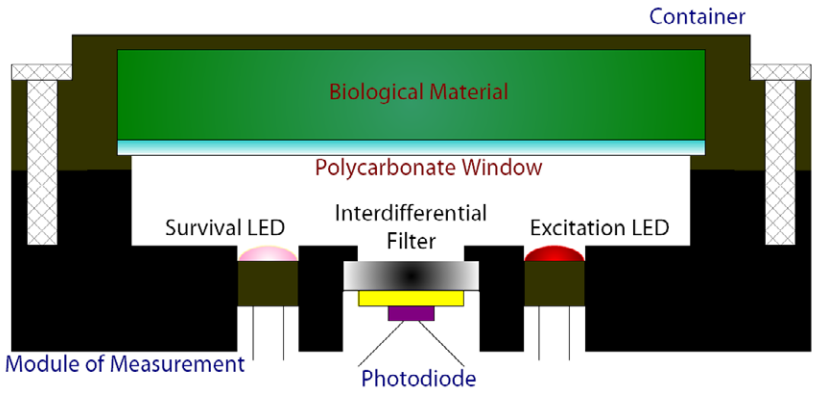

Figure 4. Schematic cross section of the measurement cell made up by two independent compartments, joined by two lateral screws: the container for the biological material on the top and the optical devices lodging at the bottom, for excitation and fluorescence emission measurement.

the temperature values for each measurement cycle. Power is supplied by internal batteries operating at 7.3–8.2 V.

Figure 6 shows a picture of the fabricated instrument with the optical modules still uncovered by the biological arrays caps: its weight is 0.250 kg without batteries and the external case, and the internal dimensions of the case lodging the electronic boards and the multiarrays are 100 mm (W) × 100 mm (H) × 150 mm (L).

4. Experimental testing

Experimental biosensing tests have been carried out by employing a set of wild-type and PSII-D1 protein mutants of the photosynthetic alga *Chlamydomonas reinhardtti*. This unicellular alga with only one chloroplast is important as a model of the nanostructured PSII complex; moreover, it can be easily modifiable at the level of PSII by site-directed mutagenesis.

Mutations have been carried out by site-specific mutagenesis procedures using the polimerase chain reaction (PCR) technique which allows one to change one or more specific

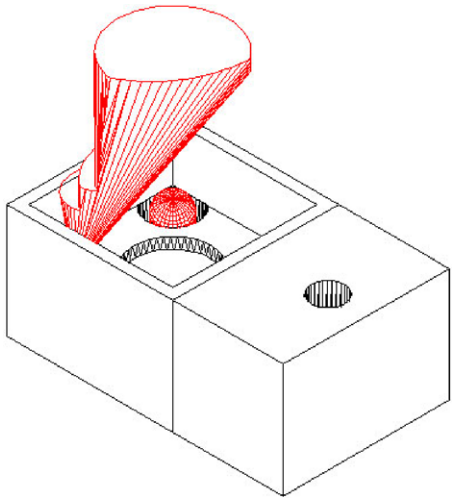

Figure 5. Simulation of the LED 'field of view' by layers: the direction points towards the centre of the upper compartment containing the biosample to be excited.

Figure 6. Picture of the instrument at the end of fabrication, showing the electronic control and measurement boards and the optical modules with the LEDs on. The biological container arrays to be placed over the optical modules are still unmounted.

nucleotides within a cloned gene, in order to create an altered form of a protein with one or more specific amino acid changes. In table 1 the names of *Chlamydomonas reinhardtti* mutants analysed in this work are listed. Amino acid abbreviations are also explained. The first letter indicates the name of the native amino acid, the central number its position in the primary

Table 1. Analysed biomediators and their mutations.

Chlamydomonas reinhardtti mutants	Targeted amino acids
S264K	S serine K lysine
A251C	A alanine C cysteine
L259I/N230Y[a]	L leucine I isoleucine N asparagine Y tyrosine
A250R	A alanine R arginine
I163N	I isoleucine N asparagine

[a] Double mutant.

polypeptide chain and the second letter the name of the amino acid after mutation. IL (D1 IntronLess of wild-type) is not listed in table 1 and represents our control biomediator.

As described in previous works [7], the 'area' parameter has been adopted in fluorescence signal deconvolution for quenching data analysis, due to the strong variations exhibited: it is defined as the area identified by the horizontal line corresponding to the maximum fluorescence value F_m and the intensity–time curve response. The output calculated by the onboard electronics, stored in the memory card and represented in the experimental response curves, is the percentage area variation with respect to the initial area value (control, baseline). A percentage of 100% corresponds to zero variation and quenching.

The biosamples were deposited by physical adsorption onto porous glass at the bottom of the biological compartments and pesticides were administrated in calibrated concentrations in the range of 10^{-10}–10^{-6} M in a pH 7.2 phosphate buffer solution to observe the response in the fluorescence curve. The following six pesticides were used: atrazine, diuron, ioxynil, terbutylazine, prometryn and linuron.

5. Results and discussions

Measurements were planned to fulfil the following main objectives: (1) the assessment of the most responsive algal mutant to each selected pesticide and in the desired concentration range; (2) study of the biomediator selectivity and identification of the minimum detectable signal; (3) study of the response curve dependence on the pesticide incubation time. Experimental results are presented in the next three subsections accordingly.

All experiments were repeated at least three times, and statistical tests were performed using analysis of variance (ANOVA). The statistical significance of the responses was evaluated by the P-level with calculated P-values $\leqslant 0.05$.

Measurement results were found to be consistent with observations recorded from previous experiments performed with the same biomediators and inhibitors by employing a commercial instrument [7]. This comparison strengthens the reliability of the performance of this new multiarray biosensor system.

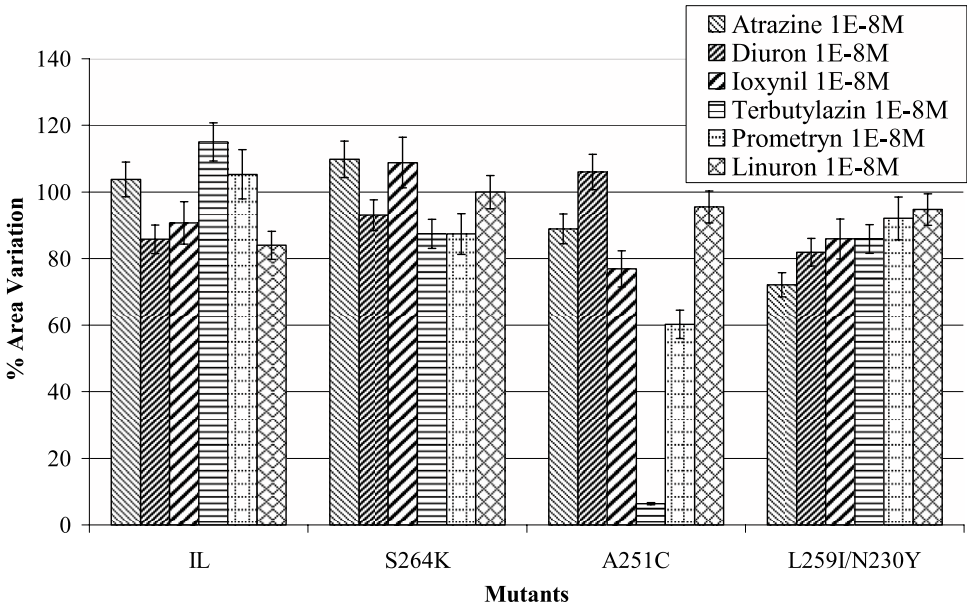

Figure 7. Measured fluorescence response of four different biosamples to six inhibitors at a fixed concentration of 10^{-8} M in a pH 7.2 phosphate buffer solution after 30 min incubation time.

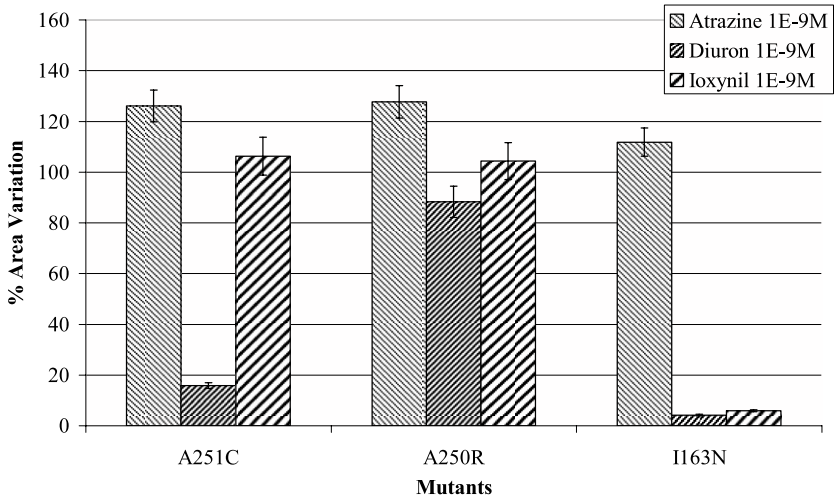

Figure 8. Measured fluorescence response of three different biosamples to three inhibitors at a fixed concentration of 10^{-9} M in a pH 7.2 phosphate buffer solution after 30 min incubation time.

5.1. Response to pesticides at different concentrations

Experimental results showing the percentage response of the six different photosynthetic biomediators are shown in figures 7 and 8 in a concentration range of the administrated inhibitor of 10^{-8} and 10^{-9} M, respectively. Figure 9 reports the response of one mutant to four different pesticide concentrations: this particular biomediator showed the most linear response with increasing concentration.

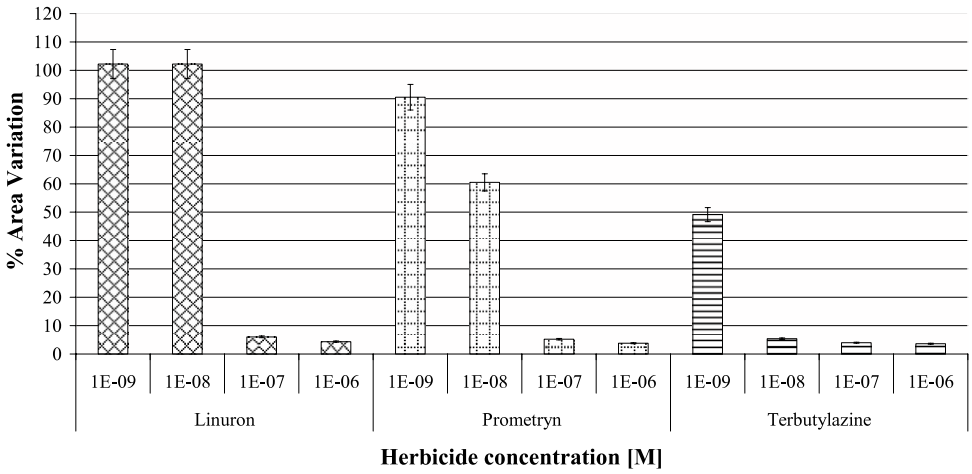

Figure 9. Measured fluorescence response of the algal mutant A251C to three inhibitors at increasing concentrations in a pH 7.2 phosphate buffer solution after 10 min incubation time.

Table 2. Selection of the most responsive algae to 10^{-8} M inhibitor concentration.

Inhibitor	Most responsive algae
Atrazine	L259I/N230Y
Diuron	L259I/N230Y
Ioxynil	A251C
Terbutylazine	A251C
Prometryn	A251C
Linuron	IL

Table 3. Selection of the most responsive mutants to 10^{-9} M inhibitor concentration.

Inhibitor	Most responsive algae
Atrazine	A250R[a]
Diuron	A251C
Ioxynil	I163N

[a] A photosynthesis activity stimulation occurs with a % area output >100%.

Tables 2 and 3 schematically summarize the results reported in figures 7 and 8 to highlight the optimal observed responses for each inhibitor at 10^{-8} and 10^{-9} M concentration. IL and S264K resulted in being the less responsive for any concentration and pesticide (except for linuron).

Very low inhibitor concentrations can cause a stimulation instead of a reduction of the photosynthesis activity of the algal mutants [11], thus producing area variations >100% in the experimental results, like some of the values represented in figures 7 and 8 (cf footnote (a) in table 3). Such high values are not due to standard deviation, which is measured to be around 5–7%.

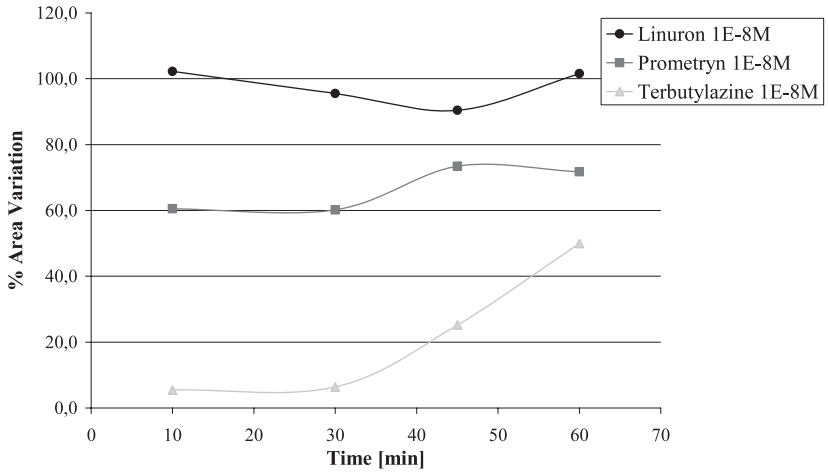

Figure 10. Fluorescence response of the mutant A251C to three inhibitors at a fixed concentration of 10^{-8} M in a pH 7.2 phosphate buffer solution measured at increasing incubation times.

5.2. Selectivity and minimum detectable signal

All tested algal mutants have proved to be sensitive to most of the pesticides. A251C is the most selective one for 10^{-8} M terbutylazine (figure 7) and 10^{-9} M diuron (figure 8), while I163N is particularly resistant to 10^{-9} M atrazine while exhibiting a high response to other pesticides (figure 8).

The minimum fluorescence signal detected by the instrument is the one emitted by 1 μg of deposited biosample and the minimum detectable inhibitor concentration is 10^{-9} or 10^{-10} M for certain classes of pesticides and particular mutants.

5.3. Response to pesticides at different incubation times

The time course of fluorescence emission in response to inhibitor treatment has been also evaluated in order to choose the appropriate measurement time and to programme the best automatic measurement cycle. Results are reported in figures 10 and 11. The mutant A251C exhibits an increasing area percentage during time in response to terbutylazine (50%) and to prometryn (15%), indicating a decrease of fluorescence signal variation. This phenomenon could be consistent with the occurrence of a physiological recovery of the photosynthetic activity of the biomediator as a feedback reaction to the pesticide. This recovery is clearly absent in the case of prometryn reported in figure 11 for the double mutant L259I/N230Y, where the output signal decreases with time (30%) as a consequence of an increase of the fluorescence emission variation: this is due to the slow chemical absorption and binding of this inhibitor molecule with the photosystem II D1 protein.

Linuron showed the lowest response variation with time ($\leqslant$10%) thus proving to be fast in binding and able to delay the alga's physiological recovery.

6. Conclusions

An innovative miniaturized multiarray fluorescence detector system has been designed and fabricated with special attention towards modularity, flexibility and low cost. The fluorescence of ten different biomediators in series can be contemporarily excited and measured under

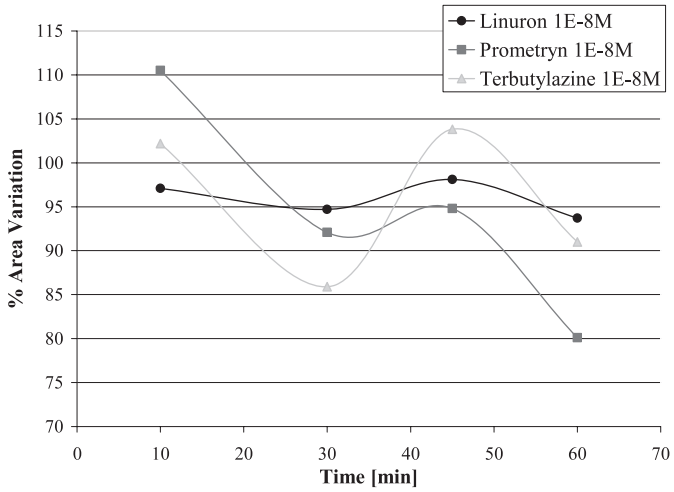

Figure 11. Fluorescence response of the mutant L259I/N230Y to three inhibitors at a fixed concentration of 10^{-8} M in a pH 7.2 phosphate buffer solution measured at increasing incubation times.

the influence of different chemical or environmental compounds, due to LED light sources and photodiodes coupled to interdifferential band pass filters, directly integrated into the optical compartment of the measurement cell. Therefore the instrument allows one to perform simultaneous and physiological multiparametric analyses. Fluorescence can be an intrinsic property of the biological material or otherwise induced by specific fluorescent probes (e.g. for DNA and proteins). Therefore, the new sensor has a large applicability in biosensor technology.

The instrument performance has been validated through several measurement campaigns in which six different photosynthetic biosamples were deposited in the cells (D1 mutants of the photosynthetic alga *Chlamydomonas reinhardtti*) and up to six pesticides were administrated (atrazine, diuron, ioxynil, terbutylazine, prometryn and linuron). Measurements were performed at different concentrations and inhibitor incubation times. The minimum fluorescence signal detected by the instrument was the one emitted by 1 μg of biomediator and the minimum detected inhibitor concentration was 10^{-9} or 10^{-10} M for certain classes of pesticides.

Experimental results proved to be comparable to previous ones obtained with a commercial apparatus, thus providing the miniaturized multiarray system with sufficient reliability to be employed in agrofood analysis and environmental monitoring.

Acknowledgments

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