Biosensors and Bioelectronics 54 (2014) 484–491

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

Microalgae dual-head biosensors for selective detection of herbicides with fiber-optic luminescent O_2 transduction

David Haigh-Flórez [a], Cristina de la Hera [b], Eduardo Costas [b], Guillermo Orellana [a,*]

[a] Chemical Optosensors and Applied Photochemistry Group (GSOLFA), Dpmt. of Organic Chemistry, Faculty of Chemistry, Complutense University of Madrid, E-28040 Madrid, Spain

[b] Microalgae Biotechnology: Production and Toxicity Group (ALBIOTOX), Dpmt. of Animal Production (Genetics), Faculty of Veterinary Medicine, Complutense University of Madrid, E-28040 Madrid, Spain

ARTICLE INFO

Article history:
Received 2 August 2013
Received in revised form
29 October 2013
Accepted 30 October 2013
Available online 7 November 2013

Keywords:
Optical biosensor
Luminescence
Microalgae
Dictyosphaerium chlorelloides
Simazine

ABSTRACT

The microalgal species *Dictyosphaerium chlorelloides* (D. c.) was immobilized into porous silicone films and their photosynthetic activity was monitored with an integrated robust luminescent O_2 sensor. The biosensor specificity towards a particular pesticide has been achieved by manufacturing a fiber-optic dual-head device containing both analyte-sensitive and analyte-resistant D. c. strains. The latter are not genetically modified microalgae, but a product of modified Luria–Delbrück fluctuation analysis followed by ratchet selection cycles. In this way the target herbicide decreases the O_2 production of the analyte-sensitive immobilized strain without affecting the analyte-resistant population response; any other pollutant will lower the O_2 production of both strains. The effect of the sample flow-rate, exposure time to the herbicide, biomass loading, biosensor film thickness, intensity of the actinic light, illumination cycle, and temperature on the biosensor response has been evaluated using waterborne simazine as test bench. The biosensing device is able to provide in situ measurements of the herbicide concentration every 180 min. The biosensor limit of detection for this herbicide was 12 $\mu g\,L^{-1}$, with a working range of 50–800 $\mu g\,L^{-1}$. The biosensor specificity to simazine has been assessed by comparing its response to that of isoproturon.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

The diminishing resources of fresh water in developed and developing countries have become a major concern during the last decades. For example, the European Union (EU) has intensified the water quality control by means of its Water Framework Directive (WFD, 2000/60/EC) or the Urban Waste Water Directive (91/271/EEC). Industrial effluents, domestic sewages or agricultural waters figure among the principal responsible of aquatic deterioration. Most of the agricultural water pollution comes from the intensive farming technologies, which use massive amounts of herbicides and fertilizers that produce soil contamination and subsequent pollution of surface and underground water (Leu et al., 2004). Herbicides are among the most extensively used chemicals in the world and constitute an important threat to the human well being (Dawson et al., 2010). Therefore, it is no surprise that, for instance, the EU has set a drinking water threshold of 0.1 $\mu g\,L^{-1}$ for each individual pesticide and 0.5 $\mu g\,L^{-1}$ for the sum of all pesticides (WFD). Other countries allow up to 4 $\mu g\,L^{-1}$ of individual pesticides. Detection of the latter pollutants is usually carried out by expensive time-consuming methods, such as, high-performance liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (GC–MS), which require highly qualified personnel for the analysis (Hogendoorn and Van Zoonen, 2000; van der Hoff and van Zoonen, 1999). The very nature of these analytical methods clash with the current scenario that demands an important additional effort to monitor *in situ*, continuously and in real time the numerous chemical substances proposed as toxicants.

Biosensors mean a step forward in the quest for early-warning analytical systems (Kissinger, 2005). They are able to carry out *in situ* on-line measurements, replacing more sophisticated, expensive and slower laboratory analytical methods (Marks et al., 2007a, 2007b). In spite of the variety of different types of bioelements (enzyme, antibody, whole-cell…) and transducers (optical, electrochemical, acoustic…) that can be used to develop a biosensor, most often the target analyte and the measuring conditions actually determine the selection of one particular type of bioelement. Whole-cell biosensors are usually more suitable for field applications than immunosensors or enzyme-based biosensors because intact cells are more tolerant to important changes in pH, temperature or ion concentrations than purified enzymes,

*: Corresponding author. Tel.: +34 913 944 220; fax: +34 913 944 103.
E-mail address: orellana@quim.ucm.es (G. Orellana).

0956-5663/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bios.2013.10.062

nucleic acids or antibodies (Belkin et al., 2010a, 2010b). Whole-cell biosensors also display other attractive features: they are cheaper to manufacture and maintain and they do not require exhaustive sample preparation. Nevertheless, whole-cell biosensors present two main disadvantages: a lower analyte specificity and longer response times than immunosensors or enzymatic biosensors.

Among the wide range of microorganisms which have been employed to develop biosensor technology (Eltzov and Marks, 2011; Lei et al., 2006), microalgae possess a number of features that suite perfectly the requirements of an early-warning environmental biosensor (Giardi and Piletska, 2006): (i) they are robust cells that can be found in vastly different environments; (ii) they are easier to isolate, grow, keep and work with than other microorganisms; (iii) and, most importantly, they are sensitive to most waterborne toxicants of interest (heavy metals, solvents, pesticides, etc.). Microalgae are specially sensitive to all those pollutants that work as inhibitors at the photosystem II (PSII) level, such as heavy metals or half of the known pesticides (Brayner et al., 2011).

As other phototrophic organisms, microalgae bear a number of protein complexes and pigments capable of capturing and transforming light into chemical energy (photosynthesis). At the PSII, chlorophyll molecules absorb photons, initiating the electron transport chain that is the cornerstone of photosynthesis. The oxidation of water molecules, also at the PSII level, replaces all the electrons lost by chlorophyll molecules, being molecular oxygen (O_2) a by-product of the whole process. However, not all those excited electrons take part in the electron transport chain: some of them simply return to their ground state with emission of light (fluorescence) and thermal dissipation (heat) (Krause and Weis, 1991). Photosynthesis inhibitors block some of the PSII light-dependant reactions, fading the photosynthetic activity (and therefore, the O_2 production), increasing chlorophyll fluorescence and raising thermal dissipation.

Most microalgal biosensors described in recent times detect and quantify those pollutants by monitoring the (proportional) O_2 production or cell fluorescence (Brayner et al., 2011). Chlorophyll fluorescence is monitored by optical transducers (Frense et al., 1998; Nguyen-Ngoc and Tran-Minh, 2007; Peña-Vazquez et al., 2009; Vedrine et al., 2003), while oxygen evolution is generally measured by electrochemical transducers (Campanella et al., 2000; Naessens and Tran-Minh, 1999; Pandard and Rawson, 1993; Shitanda et al., 2005). Microalgal biosensors based on chlorophyll fluorescence do not need an external transducer and can profit from the advantages that fiber-optic technologies have over electrical wire. They usually show a better detection limit than those based on oxygen quantification when the pollutant targets the PSII, but some of them need hours to stabilize the emission intensity before reliable measurements can be collected (Vedrine et al., 2003), and might be affected by turbid samples. Moreover, it has been demonstrated that fluorescence measurements are not the best indicator of the photosynthetic status when the toxicant of interest inhibits other target different from PSII (Olesen and Cedergreen, 2010).

Microalgal biosensors based on oxygen evolution display a swift stable response but traditional electrochemical O_2 sensors are prone to contamination and demand a frequent maintenance, an important drawback for *in situ* sensor technology (Orellana and Haigh, 2008). However, optical O_2 transducers are currently the sensors of choice for environmental and biological monitoring because they overcome those limitations and can be easily miniaturized (Orellana et al., 2011).

Regardless the transducer employed, the lack of *specificity* is a common drawback of almost every microalgae biosensor described so far, owing to the large amount of pollutants that affect the microalgae metabolism (Rogers, 1995). Some of the researchers developing microalgae biosensors have solved this problem by using a battery of different microalgae cells, on the basis that dissimilar species render distinct sensitivities to a particular toxicant (Peña-Vazquez et al., 2009; Podola et al., 2004; Tatsuma et al., 2009). Recombinant DNA technology might also provide solutions to the specificity problem: many examples of GMO (*genetically modified organisms*) biosensors can be found in the literature (Harms et al., 2006), but GMOs are not readily accepted because of the "gene flow" issue.

Another critical issue in the development of any whole-cell biosensor is the selection of an adequate solid support for the biological component. Microalgae must be immobilized onto a matrix that (i) allows their normal growth, (ii) prevents cell leaching, (iii) facilitates the access of the target analyte to the immobilized microorganisms, (iv) enhances the transducer response (O_2 permeability, fluorescence measurements), and (v) permits activation of their photosynthetic process by actinic illumination. There is a number of microalgae immobilization techniques (Moreno-Garrido, 2008), but most of them are not suitable for microalgae biosensors fabrication because they do not fulfill one or more of the above requirements.

The aim of our work was to develop a sturdy fiber-optic microalgal biosensor for robust in situ measurements of waterborne pollutants, endowed with specificity to the targeted one. To that end we have chosen interrogation of the photosynthetic status of the immobilized biomass by luminescence-based O_2 transduction and simazine as a test bench for the development of an integrated prototype for future microalgae biosensors with specificity to others pollutants. The latter must work *in situ* at those areas of special interest (waste water treatment plants, water distribution systems, environmentally protected areas, etc.). To achieve this aim, we had to (i) select the most analyte-sensitive microalgae strain and a proper way to immobilized it, (ii) develop a simazine-resistant strain without genetic modification, (iii) optimize the dual-head biosensor design, the operational conditions and the optical O_2 transducer.

The analyte selected to test the novel biosensor was simazine, a triazine that inhibits the electron transport chain at the PSII and therefore, the photosynthetic O_2 production (Wilson et al., 1999). This herbicide, widely used to control broadleaf and grassy weeds for a wide variety of crops, has a high potential to leach into ground water and run off into surface waters (Herranz et al., 2008). Simazine toxicity has been the subject of controversy between different governmental agencies: it is now banned in the European Union (91/414/EEC) but is still in use in other countries such as Japan or USA.

2. Materials and methods

2.1. Chemicals

The synthesis of the luminescent O_2 transducer, tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) dichloride (abbreviated RD3), has been described previously (Garcia-Fresnadillo et al., 1996). Neutral-cure room temperature vulcanization (RTV) silicone rubbers 3140 and 3145G used for fabricating the O_2-sensitive films were from Dow Corning (Seneffe, Belgium). Pyrogenic silica (+99.8%) was purchased from Sigma-Aldrich.

Simazine (6-chloro-*N*2,*N*4-diethyl-1,3,5-triazine-2,4-diamine) and isoproturon (3-(4-isopropylphenyl)-1,1-dimethylurea) were obtained from Riedel-de Häen (Seelze, Germany). Simazine stock solutions were prepared in DMSO (99.9%, Scharlau, Barcelona, Spain). Water used to prepare all solutions and culture media was purified with a Direct-Q system (Millipore, Bedford, MA). ImmobaSil™ porous silicone disks (10 mm diameter; 1, 0.5 and 0.25 mm

thickness) were a gift from Cellon (Bascharage, Luxembourg). The concentrated (50 ×) culture medium, BG-11 cyanobacteria freshwater solution, was purchased from Sigma-Aldrich (St Louis, MI).

2.2. Microalgae culture conditions

Dictyosphaerium chlorelloides (Naumann) Komarek and Perman (Chlorophyta) strain Dc1M and *Scenedesmus intermedius* Chodat (Clorophyta) strain Si1A were from the algae culture collection of the Albiotox research Group (Faculty of Veterinary Medicine, Complutense University of Madrid, Spain). Algae cultures were grown axenically in 500 mL Greiner flasks, using a diluted (1 ×) BG-11 solution at 20 °C with a photon irradiance of 60 μmol m^{-2} s^{-1} from white fluorescent tubes, under continuous illumination (Huertas et al., 2010). In order to maintain cultures in mid-log exponential growth, serial transfer of an inoculum (10^5 cells) to fresh medium was performed once a month. Simazine-sensitive strains (SSS) and simazine-resistant strains (SRS) were obtained, isolated and grown for both microalgae species. The protocol followed for the selection of SSS and SRS has been described previously (Orellana et al., 2009). In short, the SSS has been brought from pristine sites far away from any contamination source: *D. chlorelloides* was isolated from a mountain lake in the Sierra Nevada (southwest Spain), whereas *S. intermedius* was isolated from a pond in La'Entreuka (Sahel desert, Mauritania). The SRS microalgae were obtained from the SSS by fluctuation analysis followed by ratchet cycles (Costas et al., 2008; Huertas et al., 2010). The fluctuation analysis is able to select single mutations with high adaptive value, which confer herbicide resistance. Ratchet system ensures the occurrence of numerous spontaneous mutations with low effect followed by a strong selection pressure, which ensures the preservation of such mutations within the population.

2.3. Biosensing layers preparation

Before their colonization, the ImmobaSil disks are cut to 4 mm diameter and sterilized by autoclaving at 121 °C. The immobilization process is run in 4-well culture plates (Nalge Nunc, Roskilde, Denmark); one ImmobaSil disk is placed at the bottom of each well and covered with a 0.5, 1, 2 or 4×10^6 cell mL^{-1} solution of the selected microalgae strain in BG-11. The ImmobaSil disks bearing the microalgal colony are called henceforth *microalgal membranes*.

In this way, a set of microalgal membranes was prepared for each one of the four different strains (SSS and SRS of D.c. and S.i). The relative microalgae loading inside the ImmobaSil disks is estimated with a CCD camera (ORIEL Instaspec IV) that measures, through a 515 nm long-pass glass filter (Schott), the chlorophyll fluorescence intensity at 685 nm of the microalgal membrane in the absence of pollutants (470 nm LED excitation, Dragon Eye, Osram, Regensburg, Germany) via a bifurcated silica/silica optical fiber bundle (Polymicro Technologies, Phoenix, AZ). Moreover, no differences in the amount of chlorophyll per microalgal cell were found between the SS and SR strains of either D.c. or S.i.

2.4. O_2 sensing film preparation

An appropriate amount of 3140 silicone and pyrogenic silica (10%, w/w) was blended, spread as a thin film (125 μm), and allowed to polymerize at 70 °C for 72 h. Once cured, the film was briefly soaked into a solution of dichloromethane containing 0.25 mmol L^{-1} of RD3 indicator dye. Immediately afterwards, the dyed film was rinsed with dichloromethane and the residual solvent allowed to evaporate for 48 h. Then, the O_2 sensing film was coated on one side with a ca. 100 μm-thick layer of 3145G opaque silicone. Finally, 4 mm disks of the films were cut to sandwich them into the dual head biosensor flow-through cell (see below). Before installing the films into the biosensor chambers, they are calibrated with the electronic mass flow controllers manifold (see below).

2.5. Biosensing heads

The dual head biosensor consists of a homemade two-chamber flow cell made of aluminum, specially designed to meet the requirements of the measuring procedure (Fig. 1A). The chambers contain the SSS and the SRS microalgal membranes, respectively. A sapphire window seals one side of each flow-through chamber (Fig. 1B), allowing actinic illumination of the microalgal membrane with a blue LED (Dragon Eye, Osram, Regensburg, Germany) via the SMA-terminated bifurcated optical fiber bundle (see above). An Ophir Optronics PD200 radiometer (Har Hotzvim, Jerusalem, Israel) was used to measure the adjustable LED power (0–145 μW). A cellulose membrane (Spectrum, MWCO 14,000) is placed between the microalgal membrane and the sample flow to avoid microbial contamination of the microalgal membranes. The opposite side of the latter is separated from the O_2-sensitive film by a thin opaque silicone layer that prevents cross-talk with the actinic light and interference from the microalgae chlorophyll fluorescence (Fig. 1B). The luminescent O_2-sensing film is separated from

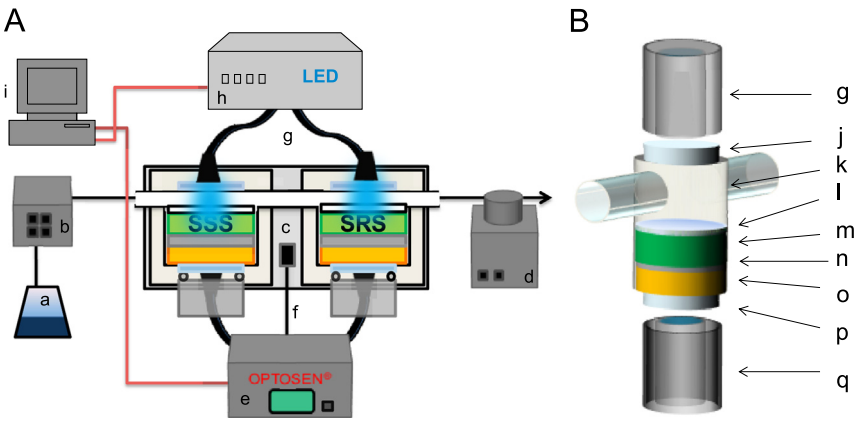

Fig. 1. (A) Schematic diagram of the microalgal dual head fiber-optic biosensor: a, water sample/culture medium; b, degasser; c, dual chamber measuring cell; d, peristaltic pump; e, optoelectronic O_2 transducer; f, temperature probe; g, optical fibers for actinic illumination; h, blue LED source; i, laptop computer. (B) Exploded view of a flow chamber: g, optical fiber for actinic illumination; j, sapphire window; k, flow-through chamber; l, cellulose membrane; m, microalgal membrane; n, gray silicone layer; o, O_2-sensitive film; p, polymer window; q, optical fiber to the optoelectronic O_2 transducer. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 1
Summary of the possible responses of the dual head microalgae biosensor for simazine monitoring.

Sample	Pollutant	*Microalgae strain*	
		SSS	*SRS*
(1) BG-11	None	$[O_2]$	$[O_2]$
(2) Water with unknown	(a) None	$\approx[O_2]$	$\approx[O_2]$
	(b) Simazine	$\downarrow[O_2]$	$\approx[O_2]$
	(c) Others	$\downarrow[O_2]$	$\downarrow[O_2]$

the optoelectronic interrogation unit by a plastic window that seals the flow-through chamber. A Peltier cell, installed under the measuring flow cell, is used to set the measuring temperature of both chambers.

2.6. Instrumentation

The luminescent O_2-sensitive film is interrogated by a fiber-optic two-channel optoelectronic transducer (Optosen®, Interlab IEC, Madrid, Spain) which performs phase-sensitive emission measurements. This portable instrument uses a high-intensity 470 nm LED as excitation source, the output of which is digitally modulated at 39 KHz (user selectable). The Optosen® unit is equipped with a temperature probe that allows automatic temperature corrections of the O_2 measurements. A peristaltic pump (MINIPULS 3, Gilson) forces samples to go through the degasser (Gastorr BG-12, FLOM, Japan) and the measuring chambers. For calibration of the oxygen transducer, an electronic mass flow controller manifold (PID Tech, Madrid, Spain) to prepare O_2/N_2 mixtures (from cylinders, Praxair, +99.995%) and a Huber Polystat K6 (Offenburg, Germany) thermostatic bath were used.

2.7. Operating protocol

The photosynthetic activity of the microalgal membranes in the dual head flow-through cell is evaluated by applying actinic light pulses of the selected duration followed by a recovery time without illumination. The O_2 level is continuously monitored by the luminescent sensor. Optimization of the illumination and dark periods is described below.

At the beginning of the sample measurement, simazine-free BG-11 medium is pumped through the biosensor until a stable analytical signal is obtained for both channels, namely when the oxygen peak heights remain constant (usually after 2 h). Then the sample is introduced in the flow-through cell and is circulated during the selected time. Once the sample is tested, the biosensor photosynthetic activity is recovered by pumping again herbicide-free BG-11 medium.

The decrease on the oxygen peak height of the SSS microalgal membrane correlates with the concentration of waterborne simazine. A calibration curve is built by plotting the percentage of photosynthetic activity inhibition of the immobilized microalgae (Eq. (1)), as a function of the simazine concentration.

$$Inhibition(\%) = 100 - (100[O_2]_s/[O_2]_m) \quad (1)$$

In Eq. (1), $[O_2]_m$ is the average value of the oxygen dissolved in BG-11 medium measured in the flow cell after the signal stabilization, and $[O_2]_s$ is the value, measured under the same conditions, of the O_2 concentration after the selected contact time of the sample with the microalgal membrane. Specificity studies with isoproturon were performed under the same conditions that those optimized for simazine.

3. Results and discussion

The main goal of this work was to overcome the shortcoming of every whole-cell biosensor, namely the lack of specificity. To achieve this aim, our biosensor materializes the concept tested by Altamirano et al. (Altamirano et al., 2004). These authors proved that different microalgae genotypes render distinct sensitivities to a particular toxicant. In this way, the dual head biosensor we have developed (Orellana et al., 2009) simultaneously monitors the O_2 production of two different (immobilized) microalgal strains. The difference between them is their opposite sensitivity to simazine: one strain (SSS) is chosen to for its high sensitivity to simazine whereas the other one (SRS) is selected for its high resistance to the herbicide (see below). The SSS provides the biosensor *sensitivity* while its *selectivity* arises from the different responses of those genotypes (Table 1). In this way, if the sample is free of pollutants, no mayor change will be observed in the O_2 production of none of the microalgal populations (Table 1.2a). However, if the sample contains a certain amount of simazine, the SSS photosynthetic activity will fade, but the microalgal strain that has been intentionally selected to be simazine resistant (SRS) will not be affected (Table 1.2b). Finally, the presence of other waterborne pollutants would lower the photosynthetic activity of both microalgal strains (Table 1.2c).

The other aim of our work has been to manufacture a robust microalgal biosensor capable of future *in situ* operation in waste water treatment plants and natural river courses. These aims have required a thorough optimization of the various parameters that contribute to the biosensor performance.

3.1. Microalgae selection and immobilization

Two different species of freshwater green algae, D.c. and S.i., were selected from a collection of more than 500 microalgae species according to their survival rate to simazine. In this way, the simazine-sensitive strains for the dual-head biosensor were obtained. Simazine-resistant strains were produced by a sequential process that starts with a modified Luria–Delbrück method followed by ratchet selection cycles. A L–D fluctuation analysis proved that SRS were the result of a rare pre-selective mutation (Marvá et al., 2010). The fact that neither the SSS nor the SRS were obtained by recombinant DNA technology would facilitate approval of the novel biosensor by the regulatory bodies.

Disks of a unique silicone sponge were used to immobilize the selected microalgae. This polymer material, in addition to being translucent to the actinic light, is extremely permeable to O_2, allowing a rapid exchange of this gas with the transducer indicator support, a non-porous silicone. Moreover, porous silicone has been used as a successful support of bacteria (Bedoya-Gutiérrrez et al., 2008; Roach et al., 2003). The attractive features of this support for whole-cell biosensor development lie on its high hydrophilicity and porosity that favor the viability and physical entrapment of microalgal cells. Three microalgae membranes of different thickness (1.0, 0.5 and 0.25 mm) attached to the same O_2 transducer film were tested for their signal intensity and response time to the photogenerated O_2 upon actinic illumination of the biomembrane in the absence of simazine. The experiments carried out showed that the thinnest microalgae membrane displays the highest sensitivity and fastest response to O_2, being the 0.25 mm-thick disks 4-fold more sensitive and 3/2-fold faster responding than the 1 mm-thick disks. For this reason, the biosensor development was carried out using always the thinnest disks as a support for the microorganism population.

Both microalgal species (D.c. and S.i.) led to SSS and SRS with similar properties in terms of sensitivity and resistance to simazine, respectively, but they displayed dramatically different

behavior with respect to the immobilization efficiency. Microalgae colonize the porous silicone matrix by sedimentation, their cells evolving as a cluster of microalgae that might favor physical encapsulation inside the pores. S.i.-populated membranes were readily cleared by the sample flow, losing almost all of their biomass concentration after a few hours of exposure to the water flow. However, D.c.-loaded membranes kept their biomass intact for months ("ship-in-the-bottle" encapsulation). For this reason, *D. chlorelloides* was the species selected to carry on the biosensor development.

Microalgal membranes loaded with SSS and SRS have proved long term stability during, at least two years of storage time. They were maintained in plastic culture plates into BG-11 medium, at room temperature (25 °C) and under periodic room lighting (light/darkness, 12/12 h).

3.2. O_2 transducer

For utmost robustness, the biosensor uses an optoelectronic device to interrogate the O_2 transducer which is based on a widely used oxygen-sensitive luminescent dye (RD3). RD3 displays a long emission lifetime (ca. 6 μs in solution under argon) and high luminescence quantum yield (ca. 0.4 in deoxygenated solution) (Orellana and Garcia-Fresnadillo, 2004). The optical principle is based on phase-sensitive detection of the indicator luminescence that avoids all the problems found for absolute emission intensity measurements, namely the signal drift originated by the indicator leaching, lamp and detector aging, and dye photo-bleaching.

The solid support selected to immobilize the RD3 molecules is a poly(dimethylsiloxane) polymer, due to its outstanding oxygen permeability. The optimization of the O_2-transducing silicone film has been the subject of another study (Lopez-Gejo et al., 2010). The opaque gray silicone outermost layer prevents excitation of the O_2 indicator dye by the blue actinic light and interference from the microalgal membrane chlorophyll fluorescence. The O_2-sensitive film must be maintained in close contact with the microalgal membrane in order to achieve the highest sensitivity with the dual-head biosensor. A typical dose-response plot of the luminescent O_2 transducer is depicted in Fig. 2.

The sensitivity of the O_2 transducer is steepest in the 0–2 mg L^{-1} range as a consequence of the heterogeneity of the indicator dye sites within the silicone film (Lopez-Gejo et al., 2010). However, the natural concentration in aerated water

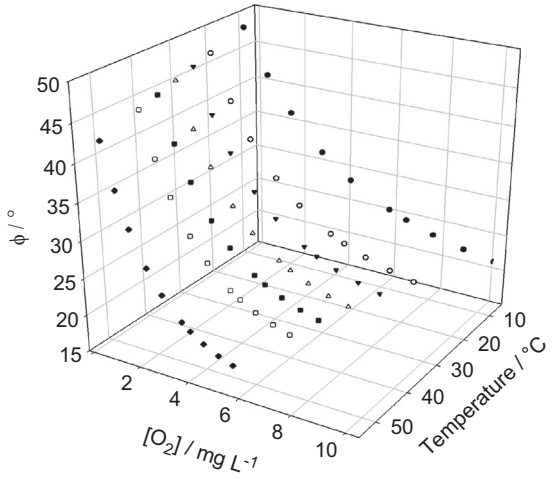

Fig. 2. Dose–response plot measured with the optoelectronic O_2 transducer as a function of temperature. The raw analytical signal provided by the device is the luminescence phase shift (ϕ), being $\tan(\phi)=2\pi f\tau$ (f is the excitation LED modulation frequency; τ is the emission lifetime of the immobilized indicator dye).

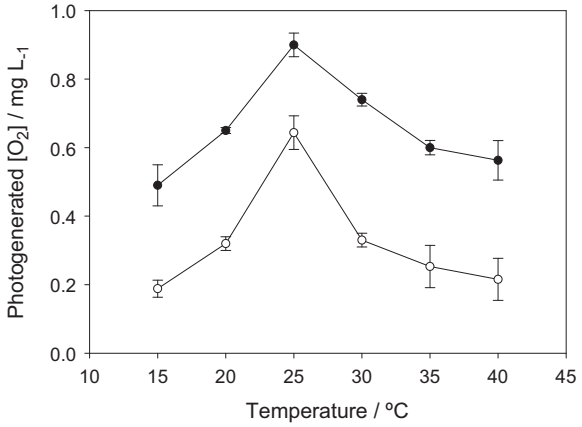

Fig. 3. Relationship between the photosynthetic activity (O_2 production) and the chamber temperature for SSS (●) and SRS (○) microalgae membranes, in the absence of pollutants. Flow rate 1 mL min^{-1}; chlorophyll emission 7,000 counts; actinic illumination power (145 μW); light/dark cycle 1.5/8.5 min; $n=5$.

samples is higher (8.24 mg L^{-1} at 25 °C and 1 atm). We have used a HPLC solvent degasser (Fig. 1A) before the water sample entrance into the measuring cell, both to work in the highest sensitivity range of the luminescent O_2 sensor and to avoid signal drifts due to fluctuations in the dissolved O_2 level. In this way, the oxygen measured continuously by the transducer comes from the microalgal photosynthesis and not from the sample. Moreover, it has been reported that removal of O_2 from the medium does not affect the microalgal photosynthesis (Cho et al., 2008).

The calibration plot is dependent on the sample temperature due to the variation of the O_2 concentration in water and to the changes of the indicator dye emission lifetime, a well known phenomenon for ruthenium(II) complexes (Van Houten and Watts, 1976). Fig. 3 also shows the dose–response curves as a function of temperature.

3.3. Effect of temperature

The biosensor response is also dependent on temperature because the microalgal photosynthesis is known to be sensitive to this parameter. Fig. 3 depicts the O_2 production of immobilized D.c. strains under different temperatures in the 15–40 °C range.

Fig. 3 demonstrates that the photosynthetic activity of the SRS membrane is lower than that of the SSS membrane. This behavior has been reported previously (Marvá et al., 2010; Sanchez-Fortun et al., 2009) and is proposed to arise from the fact that the mutations responsible for the pollutant resistance also affect negatively their photosynthetic activity and growth rate. The photosynthetic activity of both strains is favored at 25 °C; therefore, the Peltier cell installed under the dual head biosensor chambers, was set to keep the measuring temperature approximately at this value.

3.4. Effect of the biomass loading

The evaluation of the biomass loading on the sensor response was made by deposition of different solutions of microalgae at concentrations of 1, 2 and 4×10^6 cell mL^{-1}. These immobilization conditions yielded microalgal membranes with fluorescence levels of 5000, 12,000 and 23,000 counts, respectively. Microalgae membranes were prepared in quadruplicate for the SSS and SRS microalgal strains with similar fluorescence levels ($\pm$10%).

SSS membranes loaded with lower biomass concentration demonstrated to be more sensitive to simazine in the 50–300 μg mL^{-1} range, but the stability of the immobilized microalgae population over

time is more difficult to control due to higher growth rates, and they display a narrower dynamic range. Moreover, a low microalgae loading compromises the sensitivity of the O_2 transducer, particularly the one of the SRS sensor tip (see above). Membranes containing a biomass concentration that produces around 12,000 fluorescence counts provided a well-balanced situation between a good sensitivity in the SSS head together with a measurable O_2 production by the SRS membrane (Table S1).

3.5. Flow rate and analyte contact time influence

A high flow rate reduces the lag time needed to bring the sample to the microalgal membranes and the time needed to recover the O_2 base line during the dark period but, at the same time, it reduces the degasification efficiency that determines the best O_2 transducer sensitivity. Therefore, a maximum flow rate of 1 mL min^{-1} was set in agreement with the degasser manufacturer recommendations.

The physiological response of the immobilized SSS to a particular amount of simazine depends on the contact time with the toxicant. Fig. 4 shows the response of a SSS membrane exposed to two different simazine concentrations (300 and 600 μg L^{-1}) during a maximum of 50 min. Since no significative changes were observed after a 40 min exposure time, the latter was selected to perform the subsequent experiments.

3.6. Effect of the actinic light intensity and periodicity

The optimum intensity of the blue light used to stimulate the microalgae photosynthesis is closely related to the membrane biomass load: it must provide the best biological signal (O_2 production) while avoiding undesirable phenomena such as photoinhibition or photodamage of the microorganisms (Haeder et al., 1997). Different illumination powers, ranging from 20 to 145 μW at the exit of the optical fiber, were tested for different algae concentrations in the porous silicone. Finally, the maximum power available from the blue LED at the sensor tip (145 μW) proved to be the most suitable for the selected microalgal concentration in the biomembrane (12,000 counts).

The periodicity of the illumination/dark cycles was set at 10 min to allow the optoelectronic unit to collect four points during the 40 min exposure time to simazine (see Fig. 4). This measurement pattern over four points eliminates the possibility of a false positive. Different illumination/dark combinations were tested, namely 1/9, 1.5/8.5 and 3/7 min. Optimization of the cycle times relies on achieving the maximum photosynthetic O_2 peak within the most sensitive region of the luminescent transducer (see Fig. 2), and a full recovery of the initial O_2 level (Fig. S1). Both microalgal membranes were finally exposed to illumination/dark cycles of 1.5 and 8.5 min, respectively, providing a 183.6 S/N ratio.

3.7. Dual-head biosensor response

A typical response to simazine of the fiber-optic dual-head biosensor containing microalgae membranes loaded with SSS and SRS, respectively, is depicted in Fig. 5. It clearly shows the simazine inhibitory effect over the SSS photosynthetic activity and its kinetics. As expected, the SRS population is not affected by the same simazine solution. Moreover, Fig. 5 also demonstrates the recovery of the O_2 production by the SSS membrane when the simazine solution is replaced by the toxicant-free BG-11medium.

Fig. 6 shows the dose–response curves to simazine obtained with the optimized biosensor for its SSS and SRS microalgae membranes under the same measuring conditions. The height of the photogenerated O_2 peaks in the absence of simazine displays a repeatability of 1.5% (RSD, $n=10$). The biosensor limit of detection (12.2 μg L^{-1}) and the limit of quantification (40.8 μg L^{-1}) for simazine was determined, according to Morrison et al., (1979), as 3 and 10 times the standard deviation of the blank ($n=10$), respectively. However, if inhibition is considered significative above 10% (in order to leave behind the SRS range, see Fig. 6),

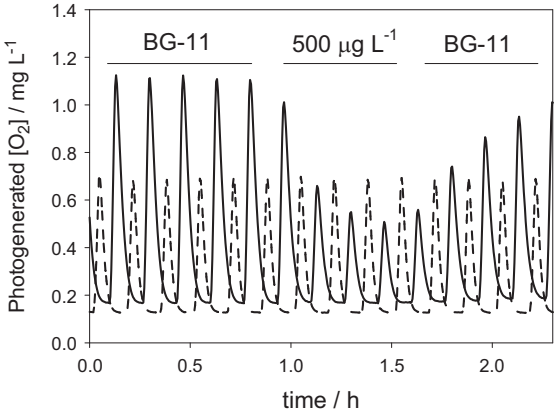

Fig. 5. Dual-head biosensor signals in the absence and the presence of 500 μg mL^{-1} simazine in BG-11 medium, for its SSS (—) and SRS (- - -) microalgae membranes; 40 min contact time, 1 mL min^{-1} flow rate, 12,000 counts algal concentration, 145 μW actinic illumination power, and 1.5/8.5 min illumination/dark cycle.

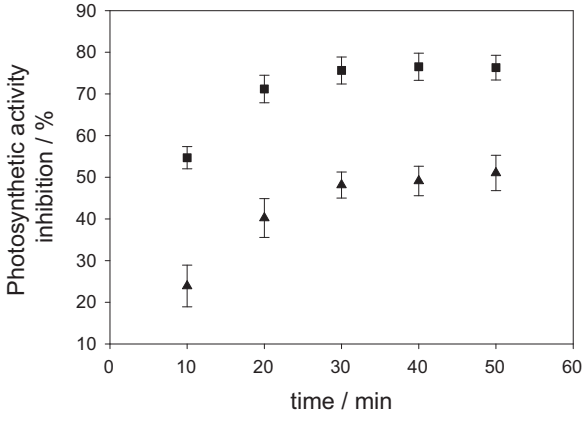

Fig. 4. Response of a SSS membrane exposed to a 300 μg L^{-1} (▲) or 600 μg L^{-1} (■) of simazine in BG-11 medium during 50 min; 1 mL min^{-1} flow rate, 12,000 counts algal concentration, 145 μW actinic illumination power, and 1.5/8.5 min illumination/dark cycle ($n=5$).

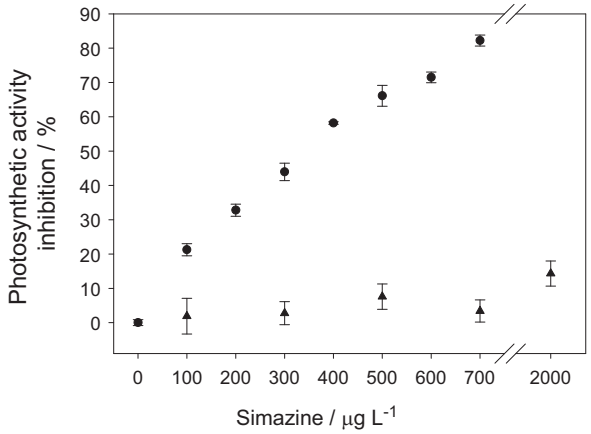

Fig. 6. Doses–response curves for the SSS (●) and SRS (▲) microalgae membranes ($n=5$); 40 min contact time, 1 mL min^{-1} flow rate, 12,000 counts algal concentration, 145 μW actinic illumination power, and 1.5/8.5 min illumination/dark cycle.

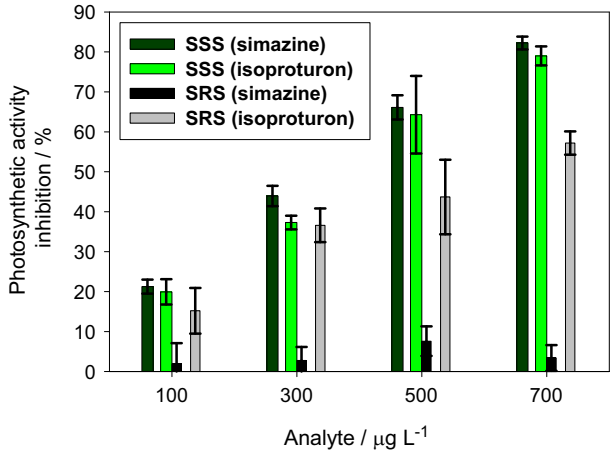

Fig. 7. Behavior of SSS and SRS microalgae membranes to different concentrations of simazine or isoproturon ($n=3$); 40 min contact time, 1 mL min^{-1} flow rate, 12,000 counts algal concentration, 145 μW actinic illumination power, and 1.5/8.5 min illumination/dark cycle.

then the quantification limit would be 52 μg L^{-1} of simazine according to Eq. (2). The analyte dynamic range for the SSS membrane extends up to 700 μg L^{-1}; much higher pollutant levels lead to irreversible damage to the immobilized SSS population. The highly resistant genotype of the ratchet-generated SRS is demonstrated by the small inhibition of their photosynthetic activity at 2000 μg L^{-1} simazine concentration (Fig. 6). The experimental data obtained with the SSS head were fitted to a modified hyperbola (Eq. (2)) with $r > 0.9978$ (SSPS SigmaPlot 11.0).

$$[\%\text{Inh}] = 116.4993\{1-\exp(-0.0017[simazine])\} \quad (2)$$

Better sensitivity is obtained by decreasing the biomass loading (Table S1) but, in that case, the robustness of the biosensor is jeopardized because of the observed reduction of the herbicide detection range and the signal drift due to the increase of the polymer-supported microalgae population.

In the laboratory, we have been continuously measuring various simazine concentrations up to 700 μg mL^{-1} (interspersed with BG-11 recovery periods) for 2 months with no noticeable signal drift. Moreover, two SSS microalgae biomembranes have been used discontinuously up to two years with successful results.

3.8. Biosensor selectivity

The selectivity to simazine of the optimized dual-head biosensor was tested using isoproturon, another widespread herbicide that also inhibits the photosynthetic activity at the PSII level and is used to protect the same crops. Fig. 7 summarizes the response of the SSS and SRS membranes to 100, 300, 500 and 700 μg L^{-1} of simazine or isoproturon in BG-11 medium. Although both pollutants target the PSII at the D1 protein (Mengistu et al., 2000; Singh et al., 2012), they interact with different amino acids of the protein, justifying why SRS mutants are not resistant to isoproturon. As anticipated, simazine-resistant mutants proved to be sensitive to isoproturon (a maximum of 55% inhibition in the concentration range tested, Fig. 7), although they displayed a lower sensitivity to isoproturon than the SSS microalgae. The latter shows a similar sensitivity to simazine than to isoproturon as a consequence of the origin of the *D. chlorelloides* strain employed (a pristine natural place where herbicides were never used). This is not the case of the resistant microalgae which, thanks to their mutated genome, show distinct sensitivity to simazine and isoproturon.

4. Conclusions

Thanks to immobilization of *D. chlorelloides* onto a unique porous silicone a robust whole-cell biosensor has been developed showing no leaching of the polymer-supported microalgae and extraordinary permeability to the photosynthetically generated O_2, which is measured with a reliable luminescent transducer. Moreover, a patented fiber-optic dual-head design allows simultaneous interrogation of tailored analyte-sensitive and analyte-resistant immobilized strains to confer the biosensor enhanced sensitivity and selectivity towards the target waterborne pollutant without having recourse to genetic engineering of the microorganism.

The thorough sensor design and operational method optimization has been carried out with the widespread herbicide simazine. Nevertheless, the limit of detection for simazine of the novel biosensor lies above the maximum limits set by most countries for the herbicide allowance (0.1–4 μg L^{-1}) in drinking water. Therefore, an online pre-concentration system would be required to develop a commercial device. Further work is in progress in our laboratory to tackle this issue for carrying out in situ real-time monitoring of waterborne pollutants, as well as to expand the usefulness of the dual-head microalgae strategy to detect and quantify other analyte species in water courses and waste water treatment plants.

Acknowledgments

The authors thank the Madrid Community Government (Grant no. CM-S-505/AMB/0374 “FUTURSEN”), the European Regional Development Fund, the European Social Fund and Complutense University-Santander (GR58-08-910072). It is currently being funded by AQUALOGY Aqua Ambiente Servicios Integrales (Spain) under the EU LIFE10 ENV/ES/000521 (“AQUATIK”) project. We are grateful to Mr. R. Fry (Cellon, LU) for the samples of ImmobaSil D disks.

Appendix A. Supplementray material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.10.062.

References

Altamirano, M., Garcia-Villada, L., Agrelo, M., Sanchez-Martin, L., Martin-Otero, L., Flores-Moya, A., Rico, M., Lopez-Rodas, V., Costas, E., 2004. Biosens. Bioelectron. 19, 1319–1323.
Bedoya-Gutiérrrez, M., Delgado-Alonso, J., Garcia-Ares, E., Luis-García, J., Orellana, G., Moreno-Bondi, M.C., 2008. PCT Pat. Appl. WO2008081060.
Belkin, S., Gu, M.B. (Eds.), 2010a. Whole cell sensing systems I-reporter cells and devices, Series on Advances in Biochemical Engineering/Biotechnology, vol. 117. Berlin-Heidelberg, Springer.
Belkin, S., Gu, M.B. (Eds.), 2010b. Whole cell sensing systems II-applications, Series on Advances in Biochemical Engineering/Biotechnology, vol. 118. Berlin-Heidelberg, Springer.
Brayner, R., Coute, A., Livage, J., Perrette, C., Sicard, C., 2011. Anal. Bioanal. Chem. 401, 581–597.
Campanella, L., Cubadda, F., Sammartino, M.P., Saoncella, A., 2000. Water Res. 35, 69–76.
Costas, E., Flores-Moya, A., Lopez-Rodas, V., 2008. New Phytol. 189, 922–932.
Cho, C.-W., Pham, T.P.T., Jeon, Y.-C., Min, J., Jung, H.Y., Lee, D.S., Yun, Y.-S., 2008. Ecotoxicology 17, 455–463.
Dawson, A.H., Eddleston, M., Senarathna, L., Mohamed, F., Gawarammana, I., Bowe, S.J., Manuweera, G., Buckley, N.A., 2010. PLoS Med. 7, e1000357.
Eltzov, E., Marks, R.S., 2011. Anal. Bioanal. Chem. 400, 895–913.
Frense, D., Muller, A., Beckmann, D., 1998. Sens. Actuators, B 51, 256–260.
Garcia-Fresnadillo, F.D., Georgiadou, Y., Orellana, G., Braun, A.M., Oliveros, E., 1996. Helv. Chim. Acta 79, 1222–1238.
Giardi, M.T., Piletska, E.V. (Eds.), 2010. Biotechnological Applications of Photosynthetic Proteins: Biochips, Biosensors and Biodevices, Landes Bioscience-Springer Science+Bussiness, Georgetown, TX.

Haeder, D.-P., Lebert, M., Flores-Moya, A., Jimenez, C., Mercado, J., Salles, S., Aguilera, J., Figueroa, F.L., 1997. J. Photochem. Photobiol., B 37, 196–202.
Harms, H., Wells, M.C., Meer, J.R., 2006. Appl. Microbiol. Biotechnol. 70, 273–280.
Herranz, S., Ramon-Azcon, J., Benito-Peña, E., Marazuela, M.D., Marco, M.P., Moreno-Bondi, M.C., 2008. Anal. Bioanal. Chem. 391, 1801–1812.
Hogendoorn, E., Van Zoonen, P., 2000. J. Chromatogr. A 892, 435–453.
Huertas, I.E., Rouco, M., Lopez-Rodas, V., Costas, E., 2010. New Phytol. 188, 478–487.
Kissinger, P.T., 2005. Biosens. Bioelectron. 20, 2512–2516.
Krause, G.H., Weis, E., 1991. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42, 313–349.
Lei, Y., Chen, W., Mulchandani, A., 2006. Anal. Chim. Acta 568, 200–210.
Leu, C., Singer, H., Stamm, C., Mueller, S.R., Schwarzenbach, R.P., 2004. Environ. Sci. Technol. 38, 3835–3841.
Lopez-Gejo, J., Haigh, D., Orellana, G., 2010. Langmuir 26, 2144–2150.
Marks, R.S., Lowe, C.R., Cullen, D.C., Weetall, H.H., Karube, I. (Eds.), 2007a. Handbook of Biosensors and Biochips, vol. 1. Wiley, New York.
Marks, R.S., Lowe, C.R., Cullen, D.C., Weetall, H.H., Karube, I. (Eds.), 2007b. Handbook of Biosensors and Biochips, vol. 2. Wiley, New York.
Marvá, F., Lopez-Rodas, V., Rouco, M., Navarro, M., Toro, F.J., Costas, E., Flores-Moya, A., 2010. Aquat. Toxicol. 96, 130–134.
Mengistu, L.W., Mueller-Warrant, G.W., Liston, A., Barker, R.E., 2000. Pest Manage. Sci. 56, 209–217.
Moreno-Garrido, I., 2008. Bioresour. Technol. 99, 3949–3964.
Morrison, G.H., Cheng, K.L., Grasserbauer, M., 1979. Pure Appl. Chem. 51, 2243–2250.
Naessens, M., Tran-Minh, C., 1999. Sens. Actuators, B 59, 100–102.
Nguyen-Ngoc, H., Tran-Minh, C., 2007. Anal. Chim. Acta 583, 161–165.
Olesen, C.F., Cedergreen, N., 2010. Pest Manage. Sci. 66, 536–542.
Orellana, G., Cano-Raya, C., López-Gejo, J., Santos, A.R., 2011. In: Wilderer, P. (Ed.), Treatise on Water Science, vol. 3. Elsevier, Oxford, pp. 221–262.
Orellana, G., Garcia-Fresnadillo, D., 2004. Springer Ser. Chem. Sens. Biosens. 1, 309–357.
Orellana, G., Haigh, D., 2008. Curr. Anal. Chem. 4, 273–295.
Orellana, G., Lopez, V., Costas, E., Haigh-Flórez, D., Maneiro, E., 2009. PCT Pat. Appl., WO2009013370.
Pandard, P., Rawson, D.M., 1993. Environ. Toxicol. Water Qual. 8, 323–333.
Peña-Vazquez, E., Maneiro, E., Perez-Conde, C., Moreno-Bondi, M.C., Costas, E., 2009. Biosens. Bioelectron. 24, 3538–3543.
Podola, B., Nowack, E.C.M., Melkonian, M., 2004. Biosens. Bioelectron. 19, 1253–1260.
Roach, P.C.J., Ramsden, D.K., Hughes, J., Williams, P., 2003. Biosens. Bioelectron. 19, 73–78.
Rogers, K.R., 1995. Biosens. Bioelectron. 10, 533–541.
Sanchez-Fortun, S., Lopez-Rodas, V., Navarro, M., Marva, F., D'Ors, A., Rouco, M., Haigh-Florez, D., Costas, E., 2009. Environ. Toxicol. Chem. 28, 1901–1905.
Shitanda, I., Takada, K., Sakai, Y., Tatsuma, T., 2005. Anal. Chim. Acta 530, 191–197.
Singh, D.V., Adeppa, K., Misra, K., 2012. J. Mol. Model. 18, 1431–1445.
Tatsuma, T., Yoshida, Y., Shitanda, I., Notsu, H., 2009. Analyst (Cambridge, U. K.) 134, 223–225.
van der Hoff, G.R., van Zoonen, P., 1999. J. Chromatogr. A 843, 301–322.
Van Houten, J., Watts, R.J., 1976. J. Am. Chem. Soc. 98, 4853–4858.
Vedrine, C., Leclerc, J.-C., Durrieu, C., Tran-Minh, C., 2003. Biosens. Bioelectron. 18, 457–463.
Wilson, P.C., Whitwell, T., Klaine, S.J., 1999. Environ. Toxicol. Chem. 18, 1462–1468.