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Highly Specific Biosensors to Herbicides, based on Sensitive- and Resistant-Mutants Microalgae

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1. Introduction

The notion that humankind has changed the biosphere led to the Nobel prize Paul Crutzen to propose, a decade ago, the new term “Anthropocene” to denote the current interval of time in the earth dominated by human driven large-scale activities (Crutzen, 2002). The massive loss of diversity, homogenization of biotas, proliferation of opportunistic species and unpredictable emergent novelties can be considered among the distinctive features of the future biosphere (Myers & Knoll, 2001). Intensive agriculture, supported by the massive use of herbicides, pesticides and compounds with biocidal activity, is a significant cause of the biodiversity crisis (Tilman, 1999). The impact of these toxic compounds on biodiversity threatens all ecosystems, being particularly significant in those characterized by a slow response to change, such as aquatic systems. Phytoplankton are responsible for about half of the global primary production, driving essential biogeochemical cycles, exporting massive amounts of carbon to deep waters and sediments in the open ocean and strongly influencing the water-atmosphere gas exchanges (Rost et al., 2008). Since these organisms represent the basis of the aquatic food web, the repercussions of the impact on phytoplankton populations will undoubtedly affect the rest of the components of the trophic web.

Step behind of the risen up of scientific and social concern regarding the environmental pollution control, developed countries have begun to take legislative actions to protect the ecosystems from chemical pollution. For the time being, the monitoring of water quality has generally relied on the collection of spot water samples followed by extraction and laboratory-based instrumental analysis. These analytical methods usually requires the use of sophisticated equipment, skilled laboratory personnel, are time consuming, expensive and difficult to adapt for fieldwork. Besides, this provides only a snapshot of the situation at the sampling time and fails to provide more realistic information due to spatio-temporal variations in water characteristics (Rodríguez-Mozaz et al., 2006).

The European Union Water Framework Directive (WFD), one of the most important pieces of environmental legislation, is likely to transform the way that determination of water quality is undertaken. Within the next few years, the implementation of the WFD will require a considerable additional monitoring effort to be undertaken and a wide range of

substances of different chemical groups to be identified. The WFD does not mandate the use of a particular set of monitoring methods, but aims at ensuring the establishment of an adequate monitoring program and encouraging the development of new technologies and more suitable methodologies allowing on-site field monitoring (Allan et al., 2006).

2. Microalgal whole cell biosensors

The need for adequate monitoring programs and early-warning procedures to detect contaminants has prompted the development of cell-based sensors as an attractive option, provided the microorganisms used as recognition bioelements are easy to isolate and manipulate (widely available, non-hazardous), their culture and maintenance is inexpensive and they provide reliable information on the presence of the target toxic agent. The essence of a whole cell biosensor is to display a cellular activity sensitive enough to stressed environments but insensitive to the physico-chemical features of the medium it operates, to the cell life cycle and to the availability of nutrients. Likewise, it should allow a smooth integration with an appropriate transducer of the biological signal.

The use of whole cells as biological recognition elements has many attractive advantages (Orellana 2008): (i) Whole-cell biosensors are usually cheap, because whole cells culturing and harvesting is easier than isolation and purification of enzymes. (ii) Whole cells are more tolerant to a significant change in pH, temperature or ionic concentration than purified enzymes. (iii) A multi-step reaction is possible because a single cell can contain all the enzymes and co-factors needed for detection of the analyte. (iv) Biosensors can easily be regenerated or maintained by letting the cells re-grow while operating in situ. (v) Extensive sample preparation is usually not required.

Green microalgae are the most preferred microorganisms to be implemented as biological element of recognition of herbicides in biosensors, since they are photosynthetic organisms. Photosynthesis inhibition estimated by chlorophyll a fluorescence of photosystem II (PSII), is an excellent indicator that rapidly reflects the toxic effect of certain pollutants. In fact, more than 50% of commercial herbicides are directed towards the direct inhibition of the electron transport at the PSII. Taking advantage of this feature, photosystem II (PSII)-based biosensors are reported to be able to detect herbicides in the environment (Giardi et al., 2001).

For example, an optical fiber based biosensor was developed for atrazine and endrine monitoring in water using *Scenedesmus subspicatus* cells, immobilized on filter paper and covered with a thin alginate layer hardened with calcium chloride (Frense et al., 1998). *Chlorella vulgaris* was immobilized at the tip of an optical fiber bundle placed inside a homemade microcell (Naessens et al., 2000), or in a rotating support holding up to five different membranes to increase the number of assays (Védrine et al., 2003), and used for the detection of herbicides affecting photosystem II (PSII) such as triazines (atrazine, simazine) or phenylureas (diuron, isoproturon) herbicides at sub- $\mu\text{g L}^{-1}$ concentration level. Nguyen-Ngoc et al. (2007) investigated the response of a microalgal biosensor to the herbicide Diuron® by measuring the variation of the chlorophyll fluorescence of *Chlorella vulgaris* strain conveniently entrapped into a sol-gel translucent support. The detection limit was $1 \mu\text{g L}^{-1}$ of diuron in water, a value much lower than the $115 \mu\text{g L}^{-1}$ limit reported with bioassays or the $10 \mu\text{g L}^{-1}$ limit reached with high performance liquid chromatography with diode array detector. The microalgae within the silica matrix kept over 95% of their initial activity after a period of 5 weeks.

To date the limiting step in the development of whole cell biosensor is the lack of specificity. Most algal biosensors have focused on reaching enough sensitivity and improving the signal measuring and the immobilization methods. Such algal biosensors are sensitive enough, although they usually present low specificity, exhibiting a summary response over a range of toxic substances. This lack of specificity represents the weak point of actual algal biosensors and is an important reason why relatively few have emerged from the laboratory to become commercially viable methods (Bengtson Nash et al., 2005). On the other hand, considering the large amount of potentially hazardous substances occurring in the environment, highly selective biosensor systems (e.g. enzyme biosensors) may also be regarded as disadvantageous (Podola et al., 2004). Ideally, combining the advantages of non-selective biosensors able to detect a variety of compounds, with a selective biosensor also allowing the identification of specific compounds would be the jump for the gap that separates academic research from field applications. In the following section we will describe recent developments directed to gain specificity in microalgae optical biosensors, focusing in the work conducted in our laboratory.

3. Theoretical and experimental setup of herbicide specific biosensors based on microalgae mutants

We have developed a new approach for increasing specificity of microalgae biosensors. This method is based in the joint use of two different genotypes from the same species, to detect a given herbicide: a highly sensitive genotype and, simultaneously, a resistant mutant to obtain high specificity.

3.1 Screening of herbicide-sensitive and –resistant genotypes

Using diverse functional and taxonomic groups, a screening of phytoplankters has been addressed to isolate strains highly herbicide-sensitive (López-Rodas et al., 2001; Costas et al., 2001; López-Rodas et al., 2007; Marvá et al., 2010; Huertas et al., 2010). The toxic effect of the herbicides has been estimated by calculating the inhibition of the acclimated maximal growth rate (m) in mid-log exponentially growing cells, in the presence of increasing concentrations of herbicides (diuron, simazine, diquat, glyphosate and others), by using the equation of Crow and Kimura (1970):

$$m = \text{Log}_e(N_t/N_0)/t$$

where N_t and N_0 are the cell numbers at the end and at the start of the experiment, respectively, and t , is the time that cultures were exposed to different doses of herbicides.

At the same time, we have applied a selection procedure maintaining large populations of dividing cells (which ensures the occurrence of mutations that confer herbicide resistance) and a strong selection pressure (which ensures the preservation of such mutations) to isolate the most herbicide-resistant genotypes. For this purpose, an experimental system based on the ratchet protocols previously described (Reboud et al., 2007; Orellana et al., 2008; Huertas et al., 2010; López-Rodas et al., this book) was applied to estimate the maximal capability for adaptation of different phytoplankton species under increasing doses of different herbicides (Table 1). The protocol aims at reaching equilibrium between strong selection intensity, by means of ratcheting to increase herbicide dose, and the maintenance of a population size

large enough to increase probability of rare spontaneous mutations that confer adaptation. These mutations occur randomly and not through specifically acquired adaptation included by herbicide (Marva et al., 2010).

From the herbicide dose-inhibition curve it is possible to calculate the lethal dose that inhibits each microalgae ancestral population (LD_{100}). The first ratchet cycle starts with three herbicide concentrations: LD_{100} , $3 LD_{100}$ and $10 LD_{100}$. Each herbicide ratchet cycle entails a threefold dose increase. Cultures must be ratcheted only up to a dose that supports population growth and are exposed to different selection levels. A ratchet cycle was concluded when no further cell growth was observed in a specific replicate after a period of 100 d. The number of ratchet cycles was therefore species dependent, as growth was a function of the ability to adapt to the selecting conditions. The experimental procedure was then applied in several independent experiments for each phytoplankton species.

Species	Habitat	Ancestral populations (before ratchet)	Derived populations (after ratchet)	Increase in adaptation (after ratchet)
<i>Emiliana huxleyi</i> (CCMP371)	Oceanic	0.10	0.15	1.5
<i>Emiliana huxleyi</i> (CCMP372)	Oceanic	0.10	0.15	1.5
<i>Emiliana huxleyi</i> (CCMP373)	Oceanic	0.05	0.15	3
<i>Isochrysis galbana</i>	Oceanic	0.10	0.15	1.5
<i>Monochrysis lutheri</i>	Oceanic	0.10	0.15	1.5
<i>Tetraselmis suecica</i>	Coastal marine	0.15	1.5	10
<i>Phaeodactylum tricornutum</i>	Coastal marine	0.10	0.45	4.5
<i>Scenedesmus intermedius</i>	Continental	0.15	40.5	270
<i>Dictyosphaerium chlorelloides</i>	Continental	0.15	13.5	90
<i>Microcystis aeruginosa</i> (3D)	Continental	0.05	0.45	9
<i>Microcystis aeruginosa</i> (6D)	Continental	0.05	0.45	9
<i>Microcystis aeruginosa</i> (7D)	Continental	0.05	0.45	9

Simazine concentrations (ppm) measured in triplicates of each strain inoculated with 1.5×10^5 cells from mid-log exponentially growing cultures and exposed to increasing simazine doses (increase interval = 0.05 ppm).

Table 1. Simazine concentration (ppm) causing 100% growth inhibition measured in ancestral populations of various phytoplanktonic organisms before the ratchet experiments as well as in derived populations after the ratchet experiments (data from Huertas et al., 2010).

Table 1 shows the maximal capability of adaptation found by Huertas et al. (2010) using diverse functional and taxonomic groups of phytoplankters (oceanic and coastal marine microalgae, and freshwater microalgae and cyanobacteria) to isolate simazine resistant strains. A simazine concentration causing 100% growth inhibition in ancestral populations

(before the ratchet experiments) is compared between the ancestral populations (before the ratchet experiments) and the derived populations (after the ratchet experiments). In spite of species diversity tested here (i.e. Cyanobacteria, Chlorophyta, Bacillariophyta, Haptophyta isolated from continental, coastal and oceanic microalgae), simazine was able to inhibit 100% growth in all ancestral populations. Adaptation of simazine at doses of 3.1 ppm (or higher) was only possible because of the occurrence of rare spontaneous simazine-resistant mutants occurring randomly during replication of organisms before exposure to simazine (Marva et al., 2010). Consequently, adaptations obtained by Huertas et al. (2010) in the ratchet protocol (i.e. up to 40.5 ppm simazine in *S. intermedius* or 13.5 ppm simazine in *D. chlorelloides*) can only be genetically achieved by new mutations that confer resistance.

3.2 Chlorophyll a fluorescence of PSII as biological signal

The approach of using selected herbicide- sensitive and –resistant microalgal mutants was tested by the development of a microalgal biosensor for the specific detection of herbicides (diuron, simazine, diquat, glyphosate and others). The inhibition of chlorophyll a fluorescence of PSII by the herbicides can be used as the biological signal.

For this purpose changes in photosynthetic activity due to herbicide exposure are estimated by measuring chlorophyll a fluorescence of PSII. Immobilized algae were placed at the tip of a bifurcated fiber-optic cable (1 m long) in a homemade flow through cell, as has been described elsewhere (Orellana et al., 2007; Pena-Vazquez et al., 2009 and cites therein), for the measurements. Algae are kept in darkness for 15 min before irradiation, during that time the sample was pumped through the flow cell. Then the biosensing layers are irradiated for 5 min with the excitation light and the fluorescence measurement is carried out immediately. Experimental signals (Fig. 1) are plotted as a function of the analyte concentration (in logarithmic scale) and the experimental data fitted to a four-parameter logistic equation (Pena-Vazquez et al., 2009):

$$I = I_{\min} + [(I_{\max} - I_{\min}) / [1 + ([Analyte]/IC_{50})^b]]$$

Changes in photosynthetic activity due to herbicide can be also estimated after 30 min of exposure, by measuring fluorescence using a pulse-amplitude modulated fluorometer PAM 2000 (Walz, Effeltrich, Germany), as explained by Schreiber et al. (1986). Maximum fluorescence of light-acclimated thylakoids (F'_m) was determined after a saturating-white light pulse, which fully close all PSII reaction centres. The inhibition of F'_m was used as an estimator of the toxic effect of herbicide, according to the formula:

$$\text{Inhibition (\%)} = 100 - [100 \times (F'_m)_{\text{herbicide}} / (F'_m)_{\text{control}}]$$

where $(F'_m)_{\text{herbicide}}$ is the maximum fluorescence after 3 min with any given herbicide concentration, and $(F'_m)_{\text{control}}$ is the maximum fluorescence of the control without herbicide after the same period of time. In all the measurements in both herbicide-exposed and control cells, the irradiance was 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. In our previous work, we have always found that maximal fluorescence of light-adapted algae (F'_m) from herbicide resistant mutants is significantly higher than that from sensitive cells.

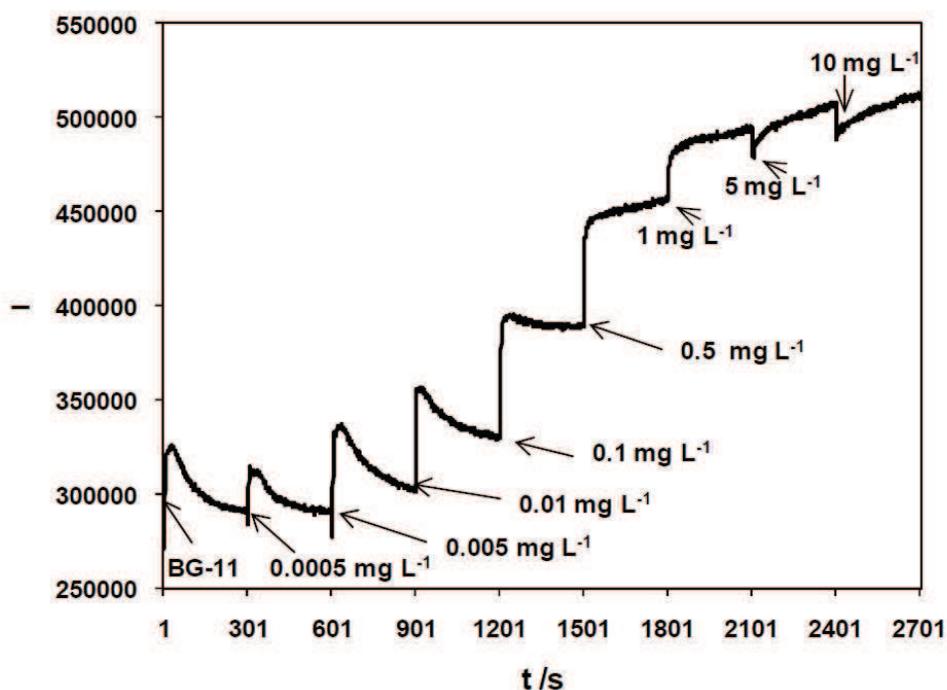


Fig. 1. Fluorescence response of a *Dictyosphaerium chlorelloides* membrane to simazine increasing concentrations in the range of 5×10^{-4} to 10 mg L^{-1} . λ_{exc} : 467 nm, λ_{em} : 699 nm. (From Peña-Vázquez et al., (2010), supplemented material).

4. Operative microalgae biosensor

Operative specific herbicide biosensors were obtained by immobilizing microalgae strains on silicone dishes (Cellon™) (Orellana et al., 2007; Orellana et al., 2008) or by encapsulating in silicate sol-gel matrices (Peña-Vázquez et al., 2009; 2010). Such procedures allow us long term stability of cells as determined by a constant $F'm$ values for at least 3 weeks. Thus, the combined measurement of $F'm$ from the two different genotypes (sensitive and resistant mutants to a given herbicide) allowed obtaining microalgae biosensor specificity (Table 2).

Microalgae mutant	Sensitive	Resistant
No herbicide present	+	+
Target herbicide present	-	+
Non-target herbicide present	-	-
+ and - denote high or low $F'm$ signal respectively		

Table 2. Selective detection of a target herbicide with a dual sensing head microalgae biosensor. (Adapted from Orellana et al., 2007).

Peña-Vázquez et al. (2009) performed representative dose response curve in the 5×10^{-4} - 10 mgL^{-1} simazine range for sensitive and resistant mutants of *Dictyosphaerium chlorelloides* (D.c.) and *Scenedesmus intermedius* (S.i.) cells immobilized in a silicate sol-gel matrix. The limit of detection was lower for the D.c. ($3.6 \text{ } \mu\text{gL}^{-1}$) than for S.i. ($31.0 \text{ } \mu\text{gL}^{-1}$) biosensors. Further research with the D.c. biosensor showed good performance regarding important criteria that may be crucial for the implementation of a marketable biosensor (Table 3).

Despite that our D.c. biosensor described above (Peña-Vázquez et al., 2009) does not reach the European Community directive for herbicide detection ($0.1 \text{ } \mu\text{gL}^{-1}$) in drinking water, when working with short irradiation times (15 min darkness/5 min irradiation), it has been demonstrated that the use of resistant strains can be a useful tool to improve biosensor specificity. In the algal biosensor wild-type sensitive cells exhibit a decrease in their biological response in presence of simazine, while resistant algae work as control exhibiting a significant smaller decrease. In contrast, under other herbicides such as atrazine or DCMU, resistant algae exhibit similar response than those of sensitive algae. This pattern demonstrates that the use of simazine-resistant cells is an appropriate procedure to improve biosensor specificity.

	D.c. biosensor
Detection limit (μgL^{-1})	3.6
IC ₅₀ (μgL^{-1})	125 ± 14
Dynamic range (μgL^{-1})	19 - 860
Response time (min)	20
Reversibility	Yes
Accuracy	High

Table 3. Response to simazine of an optical biosensor based on *Dictyosphaerium chlorelloides* (D.c.) immobilized in a sol-gel silicate flow-cell.

5. Prospective

There is a strong need for adequate monitoring programs and early-warning procedures allowing on-site field detection of herbicides polluting water reservoirs and ecosystems. Our approach based on the use of resistant strains can be a useful tool to improve microalgae biosensor specificity. In theory, our mutant selection method opens the possibility to obtain sensitive and resistant microalgae strain pairs for any target pollutant of interest, including of course, herbicides and other related pesticides, but also, other important environmental pollutants such as heavy metals, organics, novel emergent pollutants such as cosmetics and pharmaceuticals, etc. The way ahead is certainly long, but the ultimate goal of developing versatile and operative commercial equipments or biochips by means of immobilizing sensitive and resistant microalgae pairs on bifurcated fibre optic systems or multi-well plates, for example, appears realistic.

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Herbicides are much more than just weed killers. They may exhibit beneficial or adverse effects on other organisms. Given their toxicological, environmental but also agricultural relevance, herbicides are an interesting field of activity not only for scientists working in the field of agriculture. It seems that the investigation of herbicide-induced effects on weeds, crop plants, ecosystems, microorganisms, and higher organism requires a multidisciplinary approach. Some important aspects regarding the multisided impacts of herbicides on the living world are highlighted in this book. I am sure that the readers will find a lot of helpful information, even if they are only slightly interested in the topic.

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