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Optical whole-cell biosensor using *Chlorella vulgaris* designed for herbicides monitoring

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Abstract

An optical biosensor was designed for determination of herbicides as aquatic contaminants. Detection was obtained with immobilised *Chlorella vulgaris* microalgae entrapped on a quartz microfibre filter and placed in a five-membrane-home-made-flow cell. The algal chlorophyll fluorescence modified by the presence of herbicides was collected at the tip of an optical fibre bundle and sent to a fluorimeter. A continuous culture was set up to produce algal cells in reproducible conditions for measurement optimisation. Effects of flow rate, algal density, temperature, and pH on the biosensor response to atrazine were studied. Reversibility and detection limits were determined for DNOC and atrazine, simazine, isoproturon, diuron. Detection of photosystem II (PSII) herbicides was achieved at sub-ppb concentration level.

Keywords:

Biosensor; Algae; Chlorella; Fluorescence; Herbicide; Ecotoxicology

I. Introduction

Pesticides determination has been a growing concern of agriculture and health care professionals and regulatory agencies. High-performance liquid chromatography (HPLC) or gas chromatography coupled with mass spectrometry (GC-MS) have required a pre-concentration method (liquid-liquid extraction, solid-phase extraction...) for analysing pesticides in drinking water (Van der Hoff and Van Zoonen, 1999 and Hogendoorn and Van Zoonen, 2000). They demand skilled technicians, expensive equipment and are inappropriate for continuous monitoring. On the other hand, early-warning systems are needed in order to react in case of accidental pollution. Biosensors for pollutants determination have proved to be sensitive, low cost and easily adapted for on-line monitoring (Rogers, 1995). In addition, biosensors using whole cells are of ecotoxicity interest because these bioreceptors are the targets of numerous toxics.

Photosystem II (PSII)-based biosensors are reported to be able to detect herbicides in the environment (Giardi *et al.*, 2001). Photosynthesis inhibition is an interesting indicator that rapidly reflects the toxic effect of pollutants (Overnell, 1976). About 30% of herbicides are targeting the vegetal PSII (Moreland, 1980 and Draber *et al.*, 1991). They include derivatives of phenylurea, triazine, and phenolic herbicides. These substances inhibit photosynthetic

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electron flow by blocking the PSII quinone binding site and thus modify chlorophyll fluorescence.

Some biosensing systems for herbicides detection use isolated chloroplasts or intact cells of algae to measure changes in chlorophyll fluorescence (Samson and Popovic, 1988; Arsalane *et al.*, 1993; Conrad *et al.*, 1993; Yoneyama *et al.*, 1993; Merz *et al.*, 1996; El Jay *et al.*, 1997 and Van der Heever and Grobbelaar, 1998). These reagentless biosensors are compatible for in-field use or on line monitoring (Weston and Robinson, 1991; Frense *et al.*, 1998 and Naessens *et al.*, 2000). However, so far the limit of detection for atrazine reported with biosensors varied from 1 to 103 $\mu\text{g l}^{-1}$ depending on the bioreceptor used (Giardi *et al.*, 2001). The objective of this study is to design a biosensor for on-line quality monitoring of groundwater and aquatic environment. Its performances should be close to European Community directive for herbicide detection (0.1 $\mu\text{g l}^{-1}$ for a single herbicide in drinking water). The concept of algal biosensor developed by Naessens *et al.* (2000) was adopted to design a new and reliable device for herbicides determination.

II. Experimental

II.1. Solutions and reagents

Herbicides were chosen for their global or specific activity on PSII. They include atrazine [2-ethylamino-4-chloro-6-isopropyl amino-1,3,5 triazine], simazine [2,4-bis(ethylamino)-6-chloro-1,3,5-triazine], diuron [3(3,4-dichlorophenyl)-1,1 dimethylurea], isoproturon [3-(4-isopropylphenyl)-1,1-dimethylurea], and dinitro-*o*-cresol (DNOC) [4,6-dinitro-2-hydroxytoluene]. All herbicides (Pestanal®) are purchased from Riedel-de-Haen (France). They are prepared in anhydrous ethanol (Carlo Erba) to get 10^{-3} M stock solutions, which are stored in darkness at 4 °C for 1 month. All test solutions are diluted in Milli-Q® water (Millipore, France) immediately before use.

Four buffer solutions were required to investigate across the 3–10 pH range. Citrate-phosphate (pH 3–5), phosphate (pH 6–8), boric acid–borax (pH 9), borax–NaOH (pH 10) buffer solutions were used at 10 mM concentration. All chemicals were of analytical-reagent grade.

II.2. Methods of investigation

The *Chlorella vulgaris* strain (CCAP 211/12, Culture Collection of algae and protozoa, Cumbria, UK) was grown in the culture medium Lefebvre–Czarda described in the Guidance of International Organisation for Standardisation (ISO 8692). Cells were grown in a 300 ml bioreactor that enables continuous culture (1500 lx, 12 h photo-period). During illumination, the reactor was supplied with medium while culture was removed at a same flow rate (15 ml h^{-1}). Before each experiment using the biosensor, 15 ml of fresh culture was harvested 5 h after the beginning of illumination.

The active membrane was constructed by physical entrapment of the algal cells onto a porous matrix. Immobilisation was achieved by simple filtration of an algal suspension on a quartz microfibre filter QMA Whatman®. Concentration of algae in suspension was previously determined by optical density (OD) with a spectrophotometer Cary 300 (Varian, France) and counted under a microscope using a Thoma numeration cell (Polylabo, France). Typically, 20 ml of a suspension of OD=0.25 at 760 nm (about 7×10^6 cells per ml) was used for a 11.3 cm^2 membrane (3.8 cm diameter). This membrane was punched to smaller discs (8 mm in diameter, about 6×10^6 immobilised cells) that fit the five seatings of the rotating support.

II.3. Construction of the algal optical biosensor

A fibre-optic biosensor (Figure 1) was constructed with five removable algal membranes placed on a rotating support of a 10 ml homemade flow cell. An electronically controlled motor rotated the support to position the membranes alternately in front of the tip of an optical fibre bundle. The incident light hits the upper part of the membrane and the resulting fluorescence radiation is collected up to the Spex Fluorolog 2 fluorometer (from Jobin–Yvon) equipped with a microcomputer for data recording. The flow running through the biosensor is

temperature controlled using a thermostat (Lauda, Germany). Flow temperature inside the biosensor is monitored using a temperature probe (Checktemp2, Hanna instruments).

II.4. Operating conditions

Algal chlorophyll fluorescence measurement was carried out in two steps: 1 min to measure fluorescence, 4 min of darkness for the membrane to recover. While one membrane was in measurement position, the other four membranes were in stand-by position until the support rotates again. The excitation wavelength was set to 482 nm and the emission was measured at 683 nm: these wavelengths are found optimal for fluorescence determination with *C. vulgaris* (CCAP 211/12).

An Ismastec peristaltic pump (Switzerland) was used to send a continuous flow of solution through the measurement device (Figure 1) between the five algal membranes and the optical fibre bundle. The sensor was first fed with a toxic-free solution (reference solution) for 14 h to get a stable fluorescence signal. Then a herbicide was introduced to measure the biosensor response. Reversibility of the biosensor response was tested after the reference solution ran through the biosensor again for 2 h. Data were processed to obtain the mean fluorescence value (corresponding to 60 s of measurement) for each membrane.

Unless otherwise stated, all experiments were performed at 20 °C in phosphate buffer solution (pH 7, 10 mM) with a 2 ml min⁻¹ flow rate.

The biosensor long-term stability under on-line working conditions was carried out over 4 weeks. A 1 µg l⁻¹ flow solution of atrazine was tested everyday for 1 h to determine the biosensor response, then a natural water flow (Volvic, France) was performed for 23 h.

III. Results and discussion

III.1. Continuous cultivation of algal cells

A 300 ml bioreactor was designed to have a continuous culture over 5 weeks. The medium flow rate had been adjusted so that the optical density (OD) at 760 nm remained at 0.5 (ca. 14×10⁶ cells per ml). A stable physiological activity is reached 4 days after the beginning of cultivation. This system provides fresh culture for everyday consumption. Harvested cells are in a same and optimal physiological state to improve reproducibility and sensitivity of herbicides detection. Moreover, the *C. vulgaris* continuous cultivation is very easy to set up and less time consuming compare with batch cultures.

Studies conducted on life cycles of a continuous and synchronous culture of *Chlorella ellipsoidea* (Tamiya, 1964) showed that cells harvested 5 h after the beginning of illumination are most photosynthetically active since they are derived from nascent cells. Similar results demonstrated that maximal quantum efficiency of photosynthesis was achieved between 2 and 8 h after the beginning of illumination for continuous cultures of *Scenedesmus obliquus* (Senger and Bishop, 1971) and *Chlamydomonas reinhardtii* (Senger and Frickel-Faulstich, 1974). Samuelsson and Öquist (1977) have studied the DCMU-induced fluorescence at various stages of a *Chlorella pyrenoidesa* culture. They showed that maximal DCMU-induced fluorescence corresponded to a maximal photosynthesis activity. Considering these reported results, we have decided to harvest *C. vulgaris* cells 5 h after the beginning of illumination to ensure optimal physiological activity and biosensor parameters optimisation (pH, temperature...).

Additionally, the use of a continuous culture is a very convenient way to overcome problems link to culture of *C. vulgaris*.

III.2. Detection principle

Response of the algal biosensor was obtained from the chlorophyll fluorescence emission at 683 nm under 482 nm excitation light. These wavelengths were experimentally determined. Excitation maximum corresponds to the Soret band maximum absorption of chlorophyll *b* (Hipkins and Baker, 1986) present in large amounts in green algae. Emission maximum corresponds to the main emission peak (fluorescent PSII band) for *C. vulgaris* (Govindjee and Satoh, 1986) and *Scenedesmus subspicatus* (Frense *et al.*, 1998).

This biosensor has been designed from practical considerations. This five membranes configuration enables to achieve a full time use of source and detector. Membranes are under the same conditions of light and toxic exposure. It results in good repeatability in membrane fluorescence measurement (2.5% of standard deviation among five membranes using algae from the same continuous culture).

III.3. Response of the biosensor

The biosensor was tested in the absence of toxic compounds and in the presence of herbicides to compare their effects on fluorescence induction kinetics. The various steps characterising the algal biosensor response to herbicides have been described by Naessens *et al.* (2000). The presence of anti PSII herbicides (diuron, atrazine, simazine and isoproturon) increases the fluorescence emission. Reproducibility was assessed with three different continuous cultures. Five membranes of each were tested to obtain the standard deviation of the response to pollutant (6.5%). Fourteen hours were needed to reach a stable fluorescence level. This fluorescence stability is required to detect any slight modification of the signal.

Figure 2 illustrates typical fluorescence signal change following an increase in atrazine concentration in the flow stream. The signal reaches a stable plateau for each addition of atrazine, so that a calibration curve can be plotted.

The biosensor response time necessary to attain 95% maximal response was measured for flow rates comprise between 2 ml min⁻¹ and 10 ml min⁻¹ for 50 µg l⁻¹ atrazine concentration. Since a time lag is required to bring the herbicide to the bioreceptor, response times decrease with increasing carrier flow rates (data not shown). At high flow rate, the overall response time tends to approach the effective biosensor response time, which is about 10 min. This agrees with data reported by Frense *et al.* (1998).

III.4. Effect of algal density on the biosensor behaviour

The effect of algal loading in the membrane on the fluorescence signal (Figure 3) was assessed in good conditions since no leaching of algae was observed. When immobilised cells on the membrane increases, fluorescence increases and reaches a maximum corresponding to 15×10⁶ cells. Then fluorescence decreases very slightly from 15×10⁶ to 30×10⁶ cells. This is attributed to part of the fluorescence reabsorbed by neighbouring algae.

To optimise biosensor response to atrazine in the range of 0.25–10 µg l⁻¹, various concentrations of immobilised cells were tested (Figure 4). A maximal response corresponding to 6×10⁶ immobilised cells on the membrane was observed for any atrazine concentrations and also for higher concentrations (up to 1 mg l⁻¹, data not shown). The detection limit (a signal-to-noise of 3) corresponds to 0.4% of fluorescence increase. It can be conclude that the atrazine detection limit of this biosensor is 0.25 µg l⁻¹ (inset Figure 4).

III.5. Effect of temperature and pH on algal response to atrazine

Effects of temperature and pH on the algal response were tested with two different concentrations of atrazine (50 and 1000 µg l⁻¹).

The biosensor response to atrazine investigated from 10 to 30 °C (Figure 5) shows a maximum at 20 °C. This also corresponds to the temperature used for algae cultivation conditions. Good response between 10 and 25 °C still allow sensitive determination of atrazine. However, the sensor response is very affected beyond 25 °C for any atrazine concentrations and measurement in this region should be avoided.

The pHs tested ranges from 3 to 10 (Figure 6). Considering the various buffers involved, low buffer solution concentrations were used to minimise the effect of buffer composition on algae metabolism. Maximum response is achieved at pH 7, which corresponds to the culture medium pH. Between pH 5 and 9 the biosensor response is high enough to ensure sensitive determination of atrazine. Maximal response is reduced by half for pH 3 and 10. To monitor atrazine in water, the *C. vulgaris* biosensor should operate as close to the optimal conditions as possible (pH 7 and 20 °C) to achieve best results.

III.6. Long-term stability under on-line working conditions

The main objective of algae immobilisation is to stabilise the bioreceptor for the biosensor to be used repeatedly over a long period of time under on-line monitoring conditions.

No loss of activity participating to the biosensor response had been observed. The response of the biosensor to $1 \mu\text{g l}^{-1}$ of atrazine remains stable over 4 weeks (data not shown).

III.7. Detection limit and EC_{50}

One of our objectives was to improve the detection limit with a *C. vulgaris* based biosensor. Atrazine was first tested to determine optimal operating conditions, which were later applied for other herbicides. Dose response curves corresponding to atrazine, simazine, diuron and isoproturon are presented in Figure 7. Successive addition method is not appropriate to construct DNOC response curve due to its dual mode of action. The detection limit, the toxic concentration EC_{50} that affects 50% of the response and reversibility of action of these pollutants are listed in Table 1.

Triazines and phenylurea are well-known herbicides that specifically fix to the Q_B binding site of PSII with high affinity. This blocks the photosynthetic electron flow and causes an increase in chlorophyll fluorescence intensity. Experiments performed with atrazine, simazine, isoproturon and diuron (classical PSII inhibitors) showed that the biosensor was very sensitive to all four herbicides at sub ppb level. The EC_{50} obtained is consistent with the detection limit (the lower the EC_{50} , the lower the limit of detection). Those herbicides can be considered as reversible since most activity is recovered after exposition to herbicides and flushing the biosensor with a reference solution.

Phenolic herbicides have two modes of action: they are inhibitors of PSII electron transport, and also uncouplers of oxidative phosphorylation and photophosphorylation, due to their protonophoric properties (Oettmeier *et al.*, 1987). This could explain the specific behaviour of the biosensor towards the phenolic herbicide, DNOC. The fluorescence intensity increases until $300 \mu\text{g l}^{-1}$. It could be attributed to the inhibition of PSII electron transport while the uncoupling effect is comparably negligible. Although the PSII inhibition still increases at higher concentrations, the uncoupling effect of DNOC tends to become the major parameter, which decreases fluorescence signal. Although DNOC is a poor inhibitor of PSII electron transport compared with triazine, phenyl-urea and others from the same family (dinoseb, dinoterb, ioxynil) (Scalla and Gauvrit, 1991), it can be detected down to $5 \mu\text{g l}^{-1}$ (Table 1). It should be noted that the phytotoxicity of DNOC is mainly due to its uncoupling effect rather than inhibition effect on PSII electron transport (Scalla and Gauvrit, 1991).

Since its mode of action, modification of fluorescence signal is irreversible. Rinsing the biosensor with a DNOC free solution results in a decrease of the fluorescence signal far below the reference level suggesting that the membrane has been damaged due to the uncoupling effect while PSII inhibition is no longer effective. In case of herbicides monitoring, precaution should be taken in interpretation of the response particularly if DNOC concentration exceeds $300 \mu\text{g l}^{-1}$.

Conclusion

The fluorescence-based biosensor using *C. vulgaris* is particularly suitable for detection of PSII-inhibiting herbicides. It can also be used for determination of other herbicides such as DNOC, which is a poor inhibitor of PSII electron transport.

Two strategies have been adopted to improve the performance of this biosensor. First, optimisation of the bioreceptor is achieved with a continuous cultivation technique, which provides harvested cells in a same optimal physiological state that increases reproducibility. Thus it facilitates the determination of optimal operating conditions (algal density on the membrane, pH, temperature) and results in an improvement of pollutant detection. This strategy has never been reported (to our knowledge) for biosensors using *C. Vulgaris*. Second, reliability of the sensor to detect pollutants is improved as the number of assays performed is multiplied by five compared with a single membrane based biosensor. This design enables an

easier monitoring of the evolution of the flow toxicity because of the 1 min lag difference between the measurement of each membrane.

Response time and detection limit of this biosensor are compatible for groundwater and aquatic environment quality monitoring and its use as an early warning system. In fact, the level of atrazine in river water varies seasonally with the highest concentrations found in the spring and summer months (Solomon *et al.*, 1996). For example, Hall *et al.* (1993) reported detectable levels of atrazine rapidly rose to 43 $\mu\text{g l}^{-1}$ during May and then dropped sharply to 1.8 $\mu\text{g l}^{-1}$ by the end of June. The biosensor would be an appropriate tool to monitor seasonal variation of atrazine in aquatic media and be used as an alarm system. Further investigations with several micro-organisms tested simultaneously in this multi-membrane device would give a better understanding of the impact of pollutants on aqueous ecosystems.

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Figures

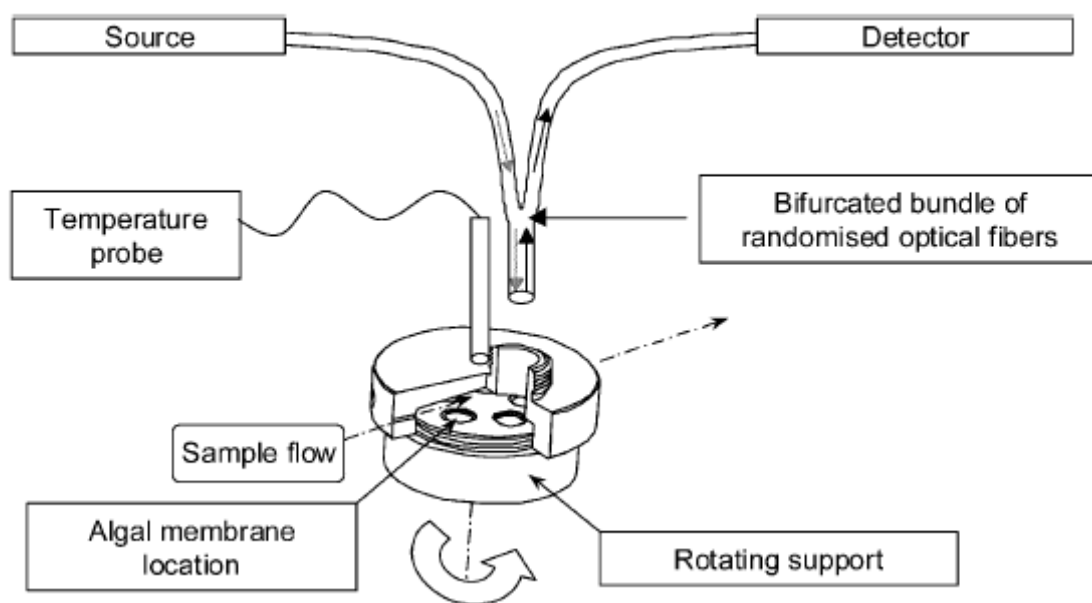


Figure 1: Experimental set-up of the algal optical biosensor for continuous toxicity assessment.

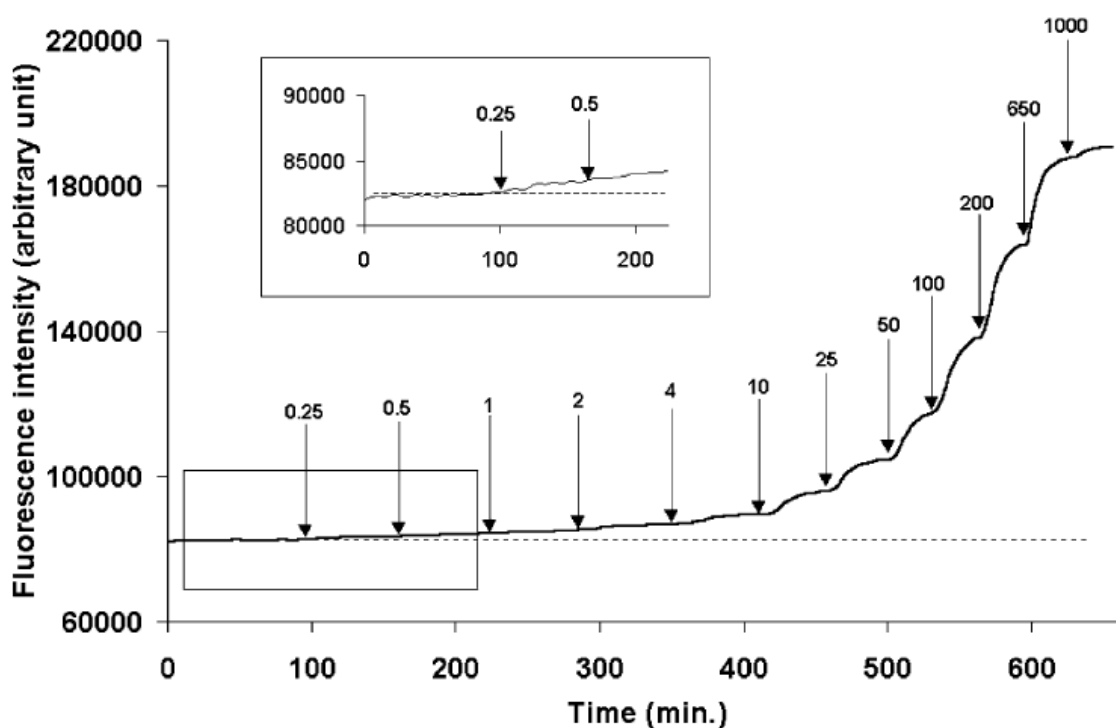


Figure 2: Typical response of a *C. vulgaris* membrane to increasing concentrations of atrazine ($\mu\text{g l}^{-1}$). Inset: response for low concentrations of atrazine, (----) fluorescence base-line. Conditions: 10 mM, pH 7.0 phosphate buffer, 20 °C, 2 ml min⁻¹ flow rate.

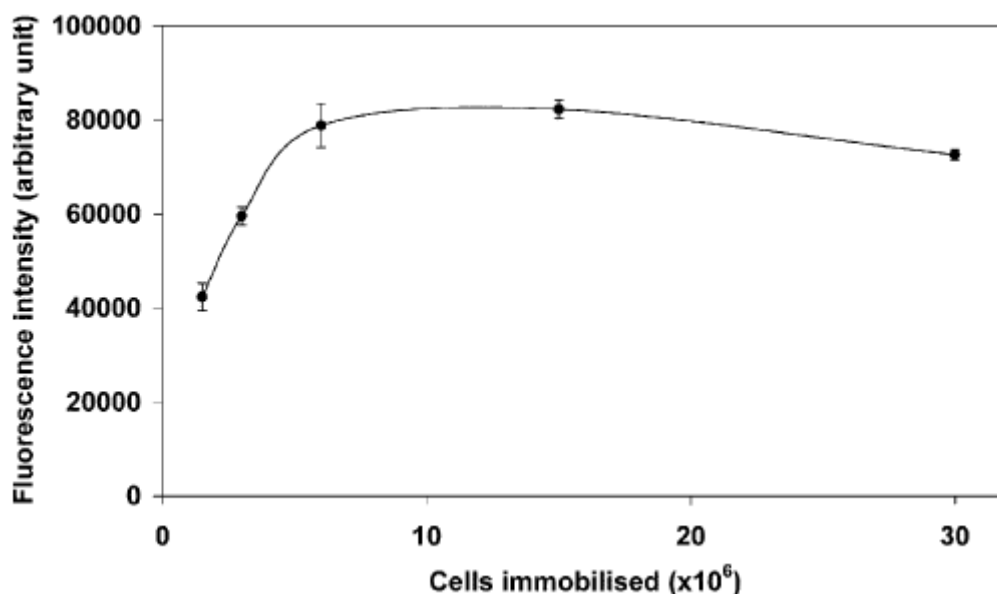


Figure 3: Effect of algal loading on chlorophyll fluorescence of *C. vulgaris*. Mean values from three membranes corresponding to three different continuous cultures were used to obtain this curve. Conditions: 10 mM, pH 7.0 phosphate buffer, 20 °C, 2 ml min⁻¹ flow rate.

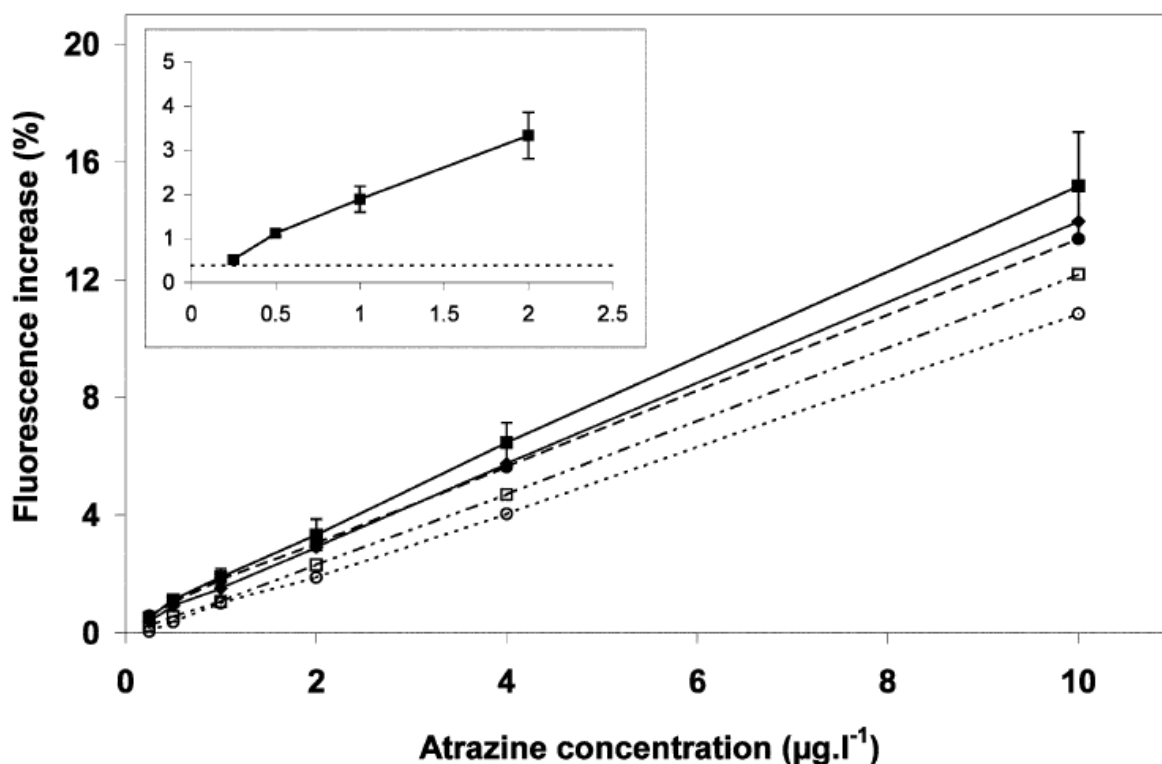


Figure 4: Effect of algal loading: 1.5 $\times 10^6$ (○), 3 $\times 10^6$ (●), 6 $\times 10^6$ (◻), 15 $\times 10^6$ (◆), 30 $\times 10^6$ (◻) cells per membrane on the sensor response to low concentrations of atrazine. Inset: response for atrazine concentration around ppb level for 6 $\times 10^6$ immobilised cells per membrane and detection limit (-----). Mean values from three membranes corresponding to three different continuous cultures were used to obtain these curves. Conditions: 10 mM, pH 7.0 phosphate buffer, 20 °C, 2 ml min⁻¹ flow rate.

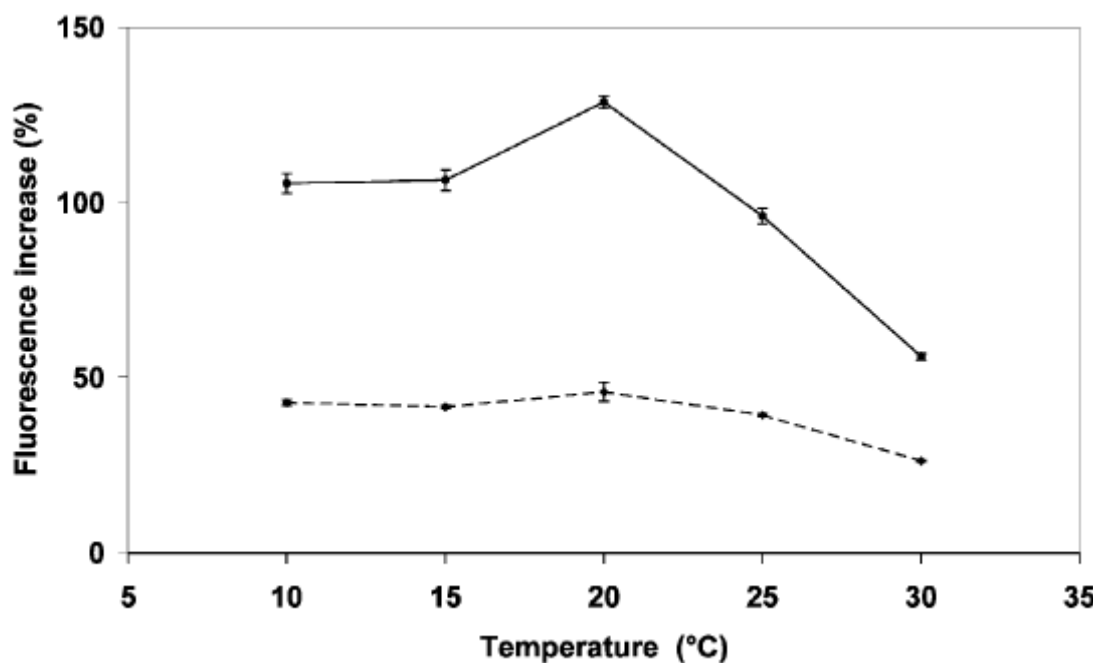


Figure 5: Effect of temperature on the sensor response to 50 µg l⁻¹ (----) and 1000 µg l⁻¹ (—) of atrazine. Mean values from three membranes corresponding to three different continuous cultures were used to obtain the curves. Conditions: 10 mM, pH 7.0 phosphate buffer, 2 ml min⁻¹ flow rate.

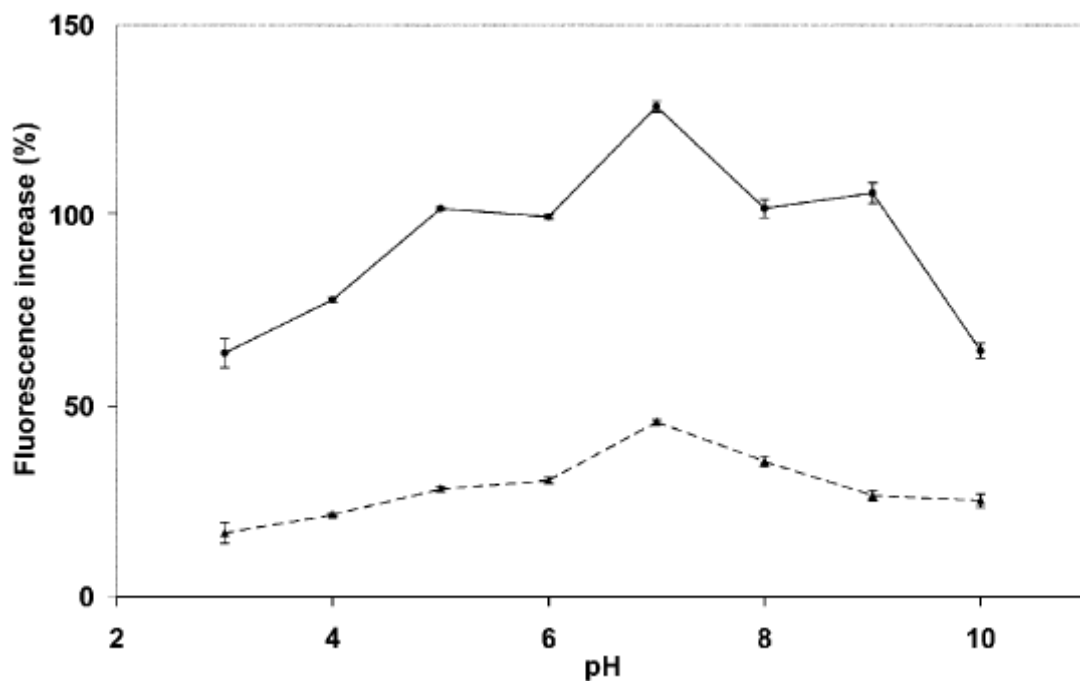


Figure 6: Effect of pH on the sensor response to 50 µg l⁻¹ (----) and 1000 µg l⁻¹ (—) of atrazine. Mean values from three membranes corresponding to three different continuous cultures were used to obtain the curves. Conditions: 10 mM, pH 3.0–5.0 citrate–phosphate buffer, pH 6.0–8.0 phosphate buffer, pH 9.0 boric acid–borax buffer, pH 10 borax–NaOH buffer, 20 °C, 2 ml min⁻¹ flow rate.

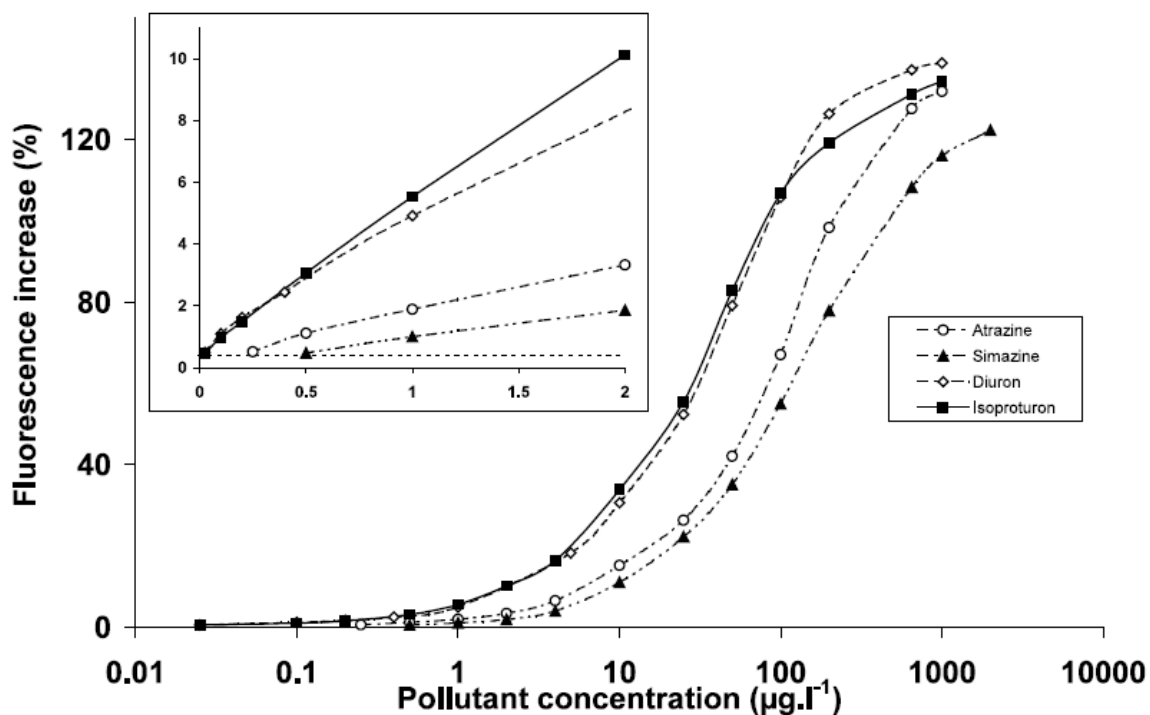


Figure 7: Dose–response curves for the detection of herbicides with chlorophyll fluorescence measurement (logarithmic scale for pollutant concentration). Inset: response for pollutant concentration (linear scale) around ppb level and detection limit (-----). Conditions: 10 mM, pH 7.0 phosphate buffer, 20 °C, 2 ml min⁻¹ flow rate.

Tables

Table 1: Characteristics of the algal optical biosensor response to different herbicides. Mean values obtained from 9 membranes (with 3 different continuous cultures)

Herbicides	Detection limit (mg l ⁻¹) (signal-to-noise of 3)	EC ₅₀ (mg l ⁻¹)	Reversibility
Atrazine	0.25	96±4	Yes
Simazine	0.5	127±4	Yes
Isoproturon	0.025	35±4	Yes
Diuron	0.025	39±4	Yes
DNOC	5	—	No