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ABSTRACT

Two algal whole cells biosensors are developed to measure specific toxicity of freshwater pollutants. Both optical and conductometric biosensors are based on inhibition of algal alkaline phosphatase (AP) and esterase activities. *Chlorella vulgaris* cells are immobilised on a membrane placed in front of an optical fiber bundle for optical sensing or deposited on the surface of an electrode for conductometric sensing. Phosphatase activity of the biosensor is strongly inhibited by heavy metal ions (60% loss of

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activity is obtained after 10 ppb Cd^{2+} and Zn^{2+} with 20 min exposure time), as equally observed with a microplate reader. Inhibition of esterase activity (EA) is actually achieved with organophosphorous pesticides such as methyl paraoxon. The biosensors exhibit a response time of about 5 min. These pollutants can be detected down to 10 ppb after being in contact with the biosensor for 30 min. The biosensor can be used up to 20 days with 90% remaining activity.

Key Words: Biosensors; Algae; Alkaline phosphatase; Esterase; Heavy metals; Pesticides; Toxicity; Early warning systems.

INTRODUCTION

The ever-growing discharge of chemicals in the environment leads to the requirement of early warning systems (EWS) to detect toxic compounds in order to react quickly in case of accidental pollution. Biosensors for pollutants determination can act as EWS thanks to their unique characteristics which include their sensitivity, low cost, and easy adaptation for on-line monitoring.[1] The aim of this study is to design two types of biosensors, optical and conductometric, based on enzyme activity inhibition directly on algal whole cells. The principle of algal biosensor[2] was adopted here to provide inexpensive biosensors for environmental control. Enzyme purification step is avoided and longer lifetime obtained with enzymes kept in their natural environment.[3,4] This technique makes use of alkaline phosphatase (AP) and esterase enzymes located on the external membrane of the whole cell *Chlorella vulgaris* for direct determination of heavy metals and pesticides. In vivo inhibition of these enzymes reflects the natural deleterious effects of toxicants on microalgae and represents a real ecological interest since algae are involved in the primary step of the food chain.

EXPERIMENTAL

Algal Cultivation

The *C. vulgaris* strain (CCAP 211/12) was purchased from The Culture Collection of Algae and Protozoa at Cumbria, United Kingdom. The axenic algal strain was grown in the culture medium and under conditions described by the International Organization for Standardization.[5]

In order to produce high AP concentration in the algal cell, the algal solution was centrifuged and the pellets were resuspended in phosphate-free

medium to starve the algal cells for 25 days which gives a maximal enzyme activity. This resuspension was carried out under the same conditions and with the same culture medium as that used previously for the growth of this strain except there were no phosphate ions. Although the medium did not contain any phosphate, an increase in cells density was observed, particularly at the beginning of the subculture period, due to the consumption of the phosphate remaining in the cell. This phosphate consumption induced an increase in AP specific activity.[6]

Esterase activity (EA) measurements were made directly on algal cells in exponential growth according to a previously published method.[7]

Construction of an Optical Biosensor

The biosensor was constructed with a removable algal membrane placed in a 1-mL home-made flow cell.[8] 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 glass microfiber filter (GF/C Whatman, 45.7-mm filter diameter, 1.2 μm pore diameter). Punching this filter has provided small disks (8 mm in diameter) which can be fitted in front of the tip of an optical fibre bundle. The incident light hit the upper part of the membrane and the resulting fluorescence radiation was collected through an optical fiber up to the Varian fluorometer equipped with a microcomputer for data recording. This device was quite suitable for the assay under flow injection analysis (FIA) conditions which required a very small amount of substrate for measurement of enzyme activity.

Determination of AP activity (APA) was carried out with methylumbelliferyl phosphate (MUP) as substrate dissolved at various concentrations in a Tris–HCl (0.1 M, pH 8.5) buffer solution containing 1 mM $MgCl_2$. The reaction product methylumbelliferone (MUF) is fluorescent. APA can easily be measured from the MUF fluorescence emission (460 nm) under excitation light (350 nm) when the MUP solution is brought into contact with the enzyme.

Determination of EA was carried out with fluoresceine diacetate (FDA) as substrate dissolved at various concentrations in a citrate (0.1 M, pH 5.4) buffer solution. The reaction product fluoresceine emits fluorescence at 538 nm under 480 nm excitation light. Both MUP and FDA were purchased from Sigma.

Construction of a Conductometric Biosensor

The conductometric transducers were fabricated at the Institute of Chemo- and Biosensorics (Munster, Germany). Two pairs of Pt (150 nm

thick) interdigitated electrodes were made by the lift-off process on Pyrex glass substrate. The central part of the sensor chip was passivated by Si_3N_4 layer to define the electrodes working area. The sensitive part of each electrode was about 1 mm^2. Measurements are based on the detection of conductance variations inside active membranes. Many enzymes can effectively catalyse consumption and production of ionic species resulting in conductivity changes.

The active membrane on the sensitive area of the first electrode was obtained by cross-linking algal cells with bovine serum albumin (BSA) in saturated glutaraldehyde (GA) vapours.[9] A mixture containing 100 μL algae solution and 10% (w/v) BSA was deposited on the sensing electrode surface using a drop method. Another mixture of 100 μL phosphate-free medium and 10% (w/v) BSA was deposited on a second electrode used as a reference. Both sensors were placed in saturated GA vapours for 30 min and then dried in air for 15–30 min.

All measurements with conductometric biosensors were carried out in daylight at room temperature with a 5 mL glass cell. Enzyme activities were measured in a stirred medium with Tris–HCl buffer (10 mM, pH 8.5) and $MgCl_2$ (1 mM) for APA and KH_2PO_4 buffer (2.5 mM, pH 8) for EA. After stabilisation of the output signal, different aliquots of the stock solution of substrate were added into the vessel. MUP (0–20 μM) and paranitrophenyl-phosphate (*p*NPP, 0–0.86 mM) were used as substrates for APA, while acetylcholine chloride (AChCl, 0–10 mM) was used as a substrate for EA. The differential output signal was recorded with a "home made" conductometric equipment[10] and the biosensor responses were plotted as a function of substrate concentration.

For toxicity assays with heavy metals, the decrease in the response to *p*NPP following 30 or 120 min exposures to Cd^{2+} or Zn^{2+} solutions (from 10 ppb to 1 ppm) was used to assess the enzyme inhibition. Measurements were carried out with 0.86 mM *p*NPP corresponding to a substrate saturation concentration. The same protocol was adopted for pesticides determination with 10 mM AChCl as substrate. The percentage of inhibition was obtained by comparing the biosensor responses dS before and after exposure to toxicants:

$$100\left(\frac{1 - \mathrm{dS}_{\text{after exposure}}}{\mathrm{dS}_{\text{before exposure}}}\right)\%.$$

RESULTS AND DISCUSSION

Responses to Substrates

APA and EA activities were determined with algae immobilized on the transducers. For APA measurements, two substrates MUP and *p*NPP were

tested to get optimal signals corresponding to the detection method. MUP was chosen for its higher sensitivity and its simplicity in optical biosensor design. This substrate was placed in a Tris buffer solution (0.1 M, pH 8.5) containing 1 mM $MgCl_2$ as an enzyme activator, and AP activity was measured in terms of fluorescence intensity from the biosensor response. Various MUP solutions were injected in the FIA system (Fig. 1): a good compromise for an acceptable response time and good repeatability was obtained with 0.5 mL injected volume and 8.4 mL min^{-1} flow rate. *p*NPP is a classical substrate for electrochemical measurements.[11] In a recent study in which a conductometric biosensor was used,[12] it has been shown that *p*NPP gave much higher conductivity variations than MUP. Therefore, *p*NPP was preferred for conductometric experiments. Fluorescence evolution (for APA) or local conductivity variations (for EA) are given in Figs. 2 and 3. Both calibration curves exhibit typical Michaelis–Menten kinetics. The relatively low response time of about 5 min observed for those substrates could be attributed to the corresponding enzymes located on the algal cell surface, so that their activity is readily available without requiring the substrates to diffuse across the cell membrane.

Determination of Heavy Metal Ions

The effects of toxic chemicals on APA and EA were tested with cadmium and lead for the optical biosensor and with cadmium and zinc for the conductometric biosensor. Enzymatic activities were determined after a definite exposure time to metal ion solutions.

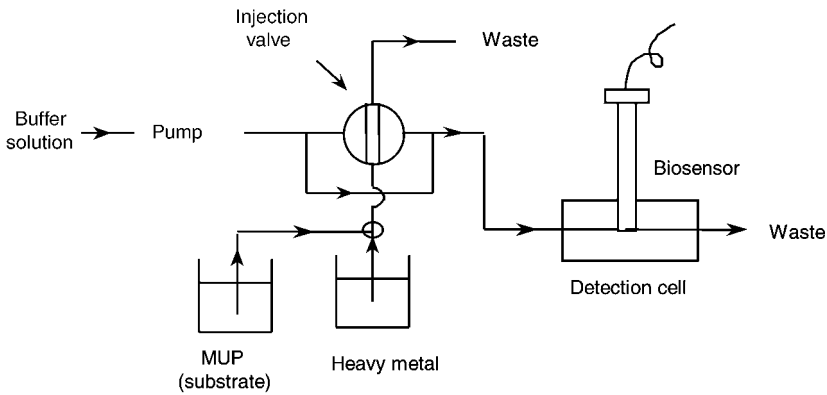

Figure 1. FIA manifold using an algal biosensor for determination of algal APA.

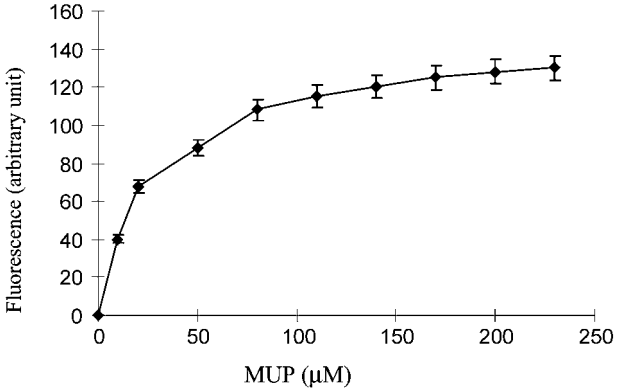

Figure 2. Response of an algal AP optical biosensor to MUP as substrate.

Fluorescence measurements with the optical biosensor was conducted with 0.1 mM MUP and 3 μM of FDA solutions which corresponds to the maximal velocity of APA and EA, respectively. When a metal ion solution was injected, APA was inhibited as indicated by the fluorescence reduction but no significant inhibition of EA was observed. Inhibition is considered as significant when the percentage of inhibition is above 15%. A comparison between the APA optical biosensor and a microplate reader based on APA inhibition showed similar results in Cd^{2+} and Pb^{2+} detection (Fig. 4).

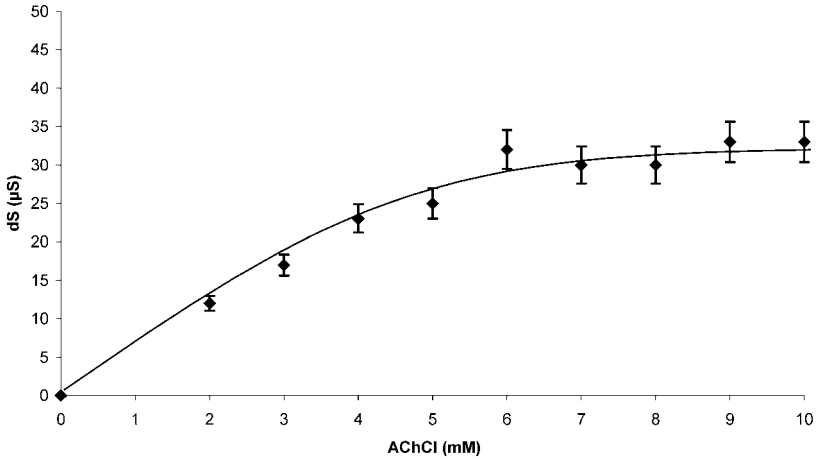

Figure 3. Response of an algal esterase conductometric biosensor to AChCl as substrate.

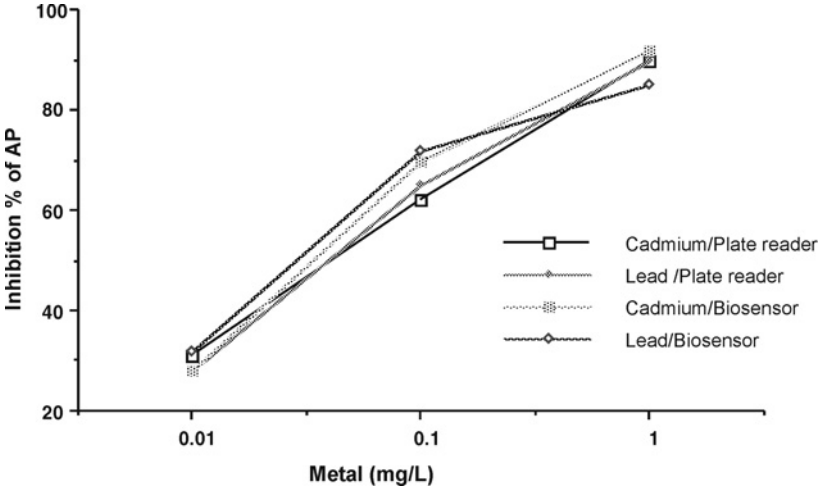

Figure 4. Comparison between micro-plate reader and optical biosensor responses to cadmium and lead concentrations in terms of percentage of inhibition (*I*%) of AP.

Conductometric biosensors based on APA inhibition were used for the determination of Cd^{2+} and Zn^{2+} (Table 1). It appears that Cd^{2+} gives higher inhibition rates than Zn^{2+} in agreement with the results obtained with both optical biosensor and microplate reader.

Since assays with a microplate reader showed that nickel, mercury, copper, and chromium are also inhibitors of *C. vulgaris* APA,[13] these compounds are now tested with our optical and conductometric biosensors. The biosensor response depends on substrate concentration for a given concentration of heavy metal ions used as inhibitors. High substrate concentrations often reduce enzyme dependence upon substrate concentration. However, such concentrations cannot be used with enzymes that are inhibited by an excess of substrate, which interferes with the metal detection also based on

Table 1. APA inhibition rates with conductometric biosensor after exposure to Cd^{2+} or Zn^{2+}.

	10 ppb		100 ppb		1 ppm	
	Cd^{2+}	Zn^{2+}	Cd^{2+}	Zn^{2+}	Cd^{2+}	Zn^{2+}
30 min	15	<10	40	25	50	30
120 min	60	60	100	90	nd	nd

enzyme inhibition. An optimal substrate concentration should then be determined from the activity–substrate concentration curve.

Although heavy metal ions also inhibit algal growth,[14] this inhibition does not interfere in this enzyme inhibition-based method because only the ratio of the peak areas in the presence and absence of heavy metals is concerned in the use of a single algal membrane.

Determination of Pesticides

Experiments were carried out with organophosphorous compounds to determine EA inhibition using conductometric biosensors. An EA inhibition of 30–40% was observed with 100 ppb of methyl paraoxon with a detection limit of 10 ppb after a 30 min contact with the enzyme. These results have to be extended to other concentrations. Since a same algal biosensor can measure both APA and EA, a multi-detection system is possible.

Toxicity Assessment of a Contaminated Effluent

In most cases, inhibition of enzymes is not really specific. The activity of a same enzyme may be affected by various types of inhibitors which could be competitive or not depending on their chemical structures.[15] This is the case for APA and EA which are also inhibited by other contaminants[15–17] and already been used to produce biosensors which could be potentiometric,[15,16] amperometric,[17] or optical.[18,19] Therefore, biosensors using those enzymes are quite adapted for assessment of overall toxicity of waste water.

The detection limits of these optical and conductometric biosensors for heavy metals indicate that they are more suitable when those compounds are present in wastewater rather than surface water. Figure 5 gives an example of APA inhibition measured with an optical biosensor after injection of an effluent containing Cd^{2+} or Zn^{2+} in the FIA carrier flow. The presence of other toxicants in effluents could interfere. In this case, the biosensor reflects a disturbance of the algal metabolism and this could be used to monitor deleterious effects of pollutants on algal cells.

Experiments using conductometric biosensors were carried out with natural effluents from French or Vietnamese rivers to determine the percentage of APA inhibition. In both cases, APA was inhibited to at least 50%, which suggests that these effluents contained APA inhibitors. The presence of heavy metal ions has been confirmed with atomic absorption spectrometry.

As for EA, APA inhibition on algal cells has been found irreversible even after several washing of the algal membrane. However, it has been reported

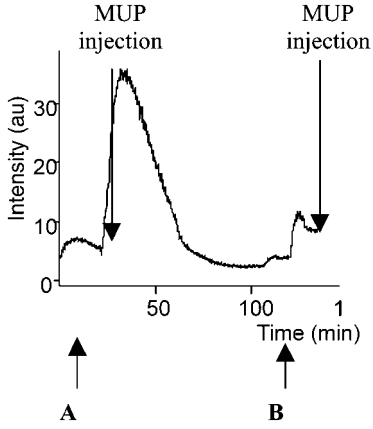

Figure 5. FIA of algal APA inhibition with optical biosensor in the abscence (A) and in the presence (B) of a toxic effluent in the carrier flow. MUP is injected in the flow as a substrate to produce the optical response.

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that purified APA could be reactivated with 10 mM EDTA.[20] When immobilized in the whole cells, this cannot be achieved and the membrane has to be changed after use. The biosensor can be used up to 20 days with 90% remaining activity.

CONCLUSION

Biosensors using enzymes present on the external membrane of the whole cells have potential advantages for determination of toxic compounds acting as APA or EA inhibitors. The enzymes remain in their natural environment which favours long-term stability and reflects the mechanism of toxic inhibition, being therefore of ecological interest. Algal AP is mainly sensitive to heavy metal ions while algal esterase is mainly sensitive to organophosphorus pesticides. If their activities are measured simultaneously, a multidetection of these compounds is possible. Such biosensors complement those based on photosynthesis inhibition designed for herbicide determination[21,22] and those based on other transducers. Optical detection has the advantage of being insensitive to electrical disturbances while conductometric detection is quite suitable for a turbid medium.

Optical and conductometric algal biosensors appear to be alternative tools to monitor pollutants in aqueous media and thus be used as EWSs. Further

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investigations with different immobilised unicellular micro-organisms, such as bacteria or yeast, would also provide insights into the effect of pollutants on aquatic ecosystems.

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