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Synchronous-scan fluorescence of algal cells for toxicity assessment of heavy metals and herbicides

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Abstract

Synchronous-scan spectrofluorometry was applied to *Chlorella vulgaris* cells to assess the toxicity of heavy metals and herbicides in water. Simultaneous scan of both the excitation and emission spectra was done at a constant wavelength difference $\Delta\lambda$ (20–140 nm) between the emission and excitation wavelengths in the range of 420–700 nm emission, where a peak of fluorescence was observed. Its position depends on $\Delta\lambda$. Fluorescence measurements were conducted with algal cells in suspension in water and immobilized in a translucent silica matrix. The influence of toxic chemicals was tested with cadmium as a heavy metal and with atrazine, diuron, DNOC and paraquat as herbicides. The toxic effect of those chemicals mainly results in a quenching of algal cells fluorescence by reducing their photosynthetic activity.

Introduction

Deterioration of the aquatic environment and groundwater contamination are usually attributed to domestic sewage, industrial effluents and agricultural products leaching from cultivated areas. Heavy metals and herbicides are among the most toxic compounds found in ground water. Cadmium is an extremely toxic metal commonly present in industrial workplaces, particularly where any ore is being processed or smelted. It is used extensively in electroplating, also found in some industrial paints and may represent a hazard when sprayed. It is also present in the manufacture of some types of batteries. Herbicides are widely used in agriculture and in landscape management, and can then be transported via surface run-off to contaminate distant water sources. Some of the herbicides in use are known to be mutagenic, carcinogenic or teratogenic. Sensitive techniques using sophisticated and expensive equipment such as gas/liquid chromatography and mass spectrometry are currently performed for their detection and monitoring. Assessment of human exposure to these toxicants through biological monitoring offers another method to evaluate the magnitude of their potential health risks. A number of bioassays have been reported for the assessment of heavy metals and herbicides toxicity. They include bacterial tests using *Photobacterium phosphoreum* (Tomulka et al., 1993), ATP bioassays (Kwan, 1989), immunoassays (Von Emon and Lopez Avilla, 1992), fish and daphnia mobility tests (Gard-Terech and Palla, 1986), enzyme assays (Cowell et al., 1995) and algal growth tests (Abou-Waly et al., 1991). Algae are essential in aquatic ecosystems since they provide oxygen and organic substances to other life forms. Chemical action of pollutants can affect their

photosynthetic activity, resulting in oxygen depletion and a decrease in aquatic biodiversity. Therefore, algae can serve as biological monitors of water quality and as biological indicators in the assessment of environmental impact of pollutants. For algal tests, microalgae in suspension have so far been used, and fluorescence detection is often preferred over growth measurement because of its higher sensitivity (Samson and Popovic, 1988; Conrad et al., 1993; Juneau et al., 2002; Eullaffroy and Vernet, 2003; Fai et al., 2007). Fluorescence spectroscopy is one of the most promising techniques of increasing importance for complex analysis (Oldham et al., 2000). Synchronous-scan spectroscopy has been used to characterize water-soluble organic compounds in atmospheric aerosols (Duarte et al., 2004), to study the interaction of Chlorella vulgaris with Fe(III) and humic acid (Liu et al., 2005; Deng et al., 2005), for classification of edible oils (Sikorska et al., 2005) and for determination of proteins (Wang et al., 2004) and human serum albumin (Hou et al., 2007). Among the benefits of synchronous-scan-fluorescence spectroscopy are its enhanced selectivity and its high sensitivity to a wide array of analytes, and no reagents are required in the case of algal cells. The aim of this paper is not to investigate the mechanism of algae photosynthesis and their photosystems which require specific techniques such as pulse amplitude-modulated (PAM) fluorometry (Jones et al., 1999), and fast repetition rate (FRR) fluorometry (Lombardi et al., 2000). In this study, synchronous-scan spectrofluorometry was applied to *C. vulgaris* cells for analytical applications and in order to assess the toxicity of heavy metals and herbicides in water.

Materials and methods

Culture of *C. vulgaris*

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 Lefebvre-Czarda and under conditions described by the International Organization for Standardization ISO 8692 (2002). Precultures were carried out at ambient temperature. Cultures started at 22 °C with a cell concentration of 10^4/mL under air bubbling (60 L/h) and periodic illumination (16 h photoperiod under 50 μE/m² s with cool white fluorescent tubes).

Choice of toxic chemicals

Cadmium (Cd II) was chosen as an example of a toxic metal for the toxicity test. It was used in the form [$Cd(NO_3)_2$,·$4H_2O$] and purchased from Merck. The following herbicides were tested because they are commonly used for their global or specific activity in cereal culture. They include DNOC [4,6-dinitro-*o*-cresol], paraquat [1,1′-dimethyl-4,4′-bipyridiniumdichloride hydrate], diuron (DCMU) [3(3,4-dichlorophenyl)1,1 dimethylurea], and atrazine [2-ethylamino-4-chloro-6-isopropyl amino-1,3,5 triazine]. Paraquat and DNOC are known as inhibitors of photosystem I (PSII) and diuron and atrazine are inhibitors of photosystem II (PSII) in plants. All herbicides were of analytical grade (Pestanal) and were purchased from Riedel-de-Haen. Herbicides were prepared in Milli-Q water (Millipore) to obtain stock solutions which are stored in darkness at 4 °C for 7 days. All test solutions were diluted in Milli-Q water immediately before use.

Cell entrapment

Sodium silicate (0.4 M, 4 mL) and LUDOX (8.5 M, 4 mL) solutions were thoroughly mixed to obtain a homogeneous silica solution. An HCl, 4 M solution was then added drop by drop until pH 7 was reached to induce the gelation process. An algal solution at various concentrations was immediately introduced under stirring and the resulting solution was cast in cuvettes to produce silica matrixes containing algal cells. Gelation was obtained in a few minutes. To assess its toxicity to algal cells, a heavy metal or herbicide at different

concentrations was added to the algal solution just before the gelation process. All experiments were performed at ambient temperature.

Synchronous-scan spectrofluorometry

Synchronous-scan fluorometry was performed with a Spex Fluorolog 2 spectrofluorometer from Jobin–Yvon equipped with a microcomputer for data recording. Fluorescence intensity was recorded, while excitation and emission wavelengths were synchronously scanned with two monochromators. Assays were carried out in cuvettes with algal cells either in solution or entrapped in a silica matrix. Illumination was obtained with an excitation/emission slit of 10/10 nm under a PMT voltage of 950 V: these parameters ensured reproducible conditions to induce fluorescence. Various $\Delta\lambda$ ranging from 20 to 140 nm were maintained between excitation and emission wavelengths during synchronous scan which was conducted with an increment of 0.5 nm and an integration time of 0.1 s. Fluorescence intensity is expressed in arbitrary unit (au). All experiments were done in triplicate.

Results

Synchronous-scan spectroscopy of *C. vulgaris* in an aqueous suspension

Synchronous-scan spectroscopy is a technique wherein simultaneous scan of both the excitation and emission spectra is done at a constant difference between the emission and excitation wavelengths, $\Delta\lambda=\lambda_{em}-\lambda_{ex}$. This technique was first carried out with algal cells in suspension in water and the fluorescence emission recorded in the range of 420–700 nm, where a peak was observed (Fig. 1). The position of the peak depends on the wavelength difference $\Delta\lambda$: the greater the $\Delta\lambda$, the lower the fluorescence emission wavelength corresponding to the peak. To $\Delta\lambda$=20 nm corresponds a peak at 670 nm, while to $\Delta\lambda$=140 nm corresponds the peak located at 540 nm. The highest emission peak corresponds to $\Delta\lambda$=20 nm, which is thus chosen for toxicity assessment. When cadmium is added to the algal suspension, the peak height is reduced. An important reduction of the peak is observed at 670 nm with 16 ppb cadmium after 1 h incubation time. These conditions are used to obtain the algal cells spectra in the presence of cadmium.

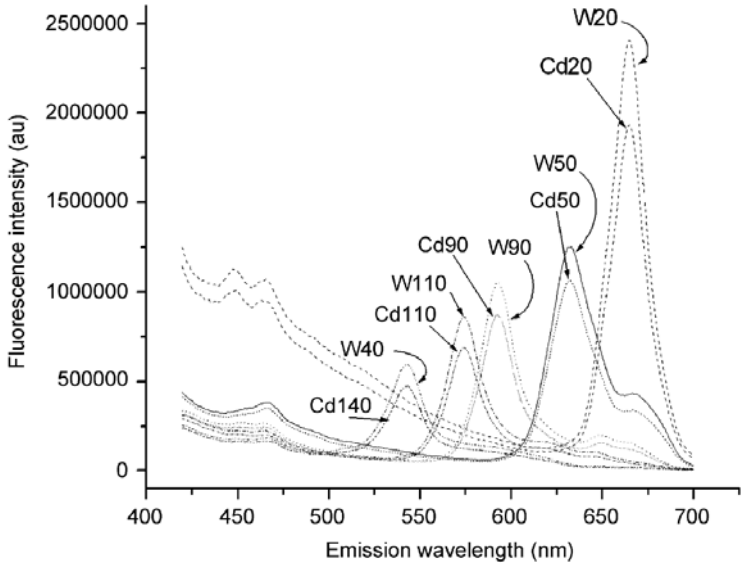

Fig 1: Synchronous fluorescence spectroscopy of "Chlorella vulgaris" cells in suspension in water (35×10⁶ cells/mL, pH 7) in the absence (W) and in the presence (Cd) of cadmium (16 ppb) using various wavelength differences (Δλ=20–140 nm) between excitation and emission wavelengths. Fluorescence intensity is expressed in arbitrary unit (au).

Synchronous fluorescence spectroscopy of *C. vulgaris* immobilized in a translucent matrix

Fluorescence measurements are not reliable when samples vary in homogeneity. The use of microalgae suspensions presents the problem of maintaining a homogeneous sample (Cosgrove and Borowitzka, 2006). Immobilization in a silica matrix provides a simple solution to this problem. However, immobilization of algal cells by physical entrapment may affect their physicochemical properties (Bozeman et al., 1989). Therefore, it is necessary to compare their behavior before and after entrapment. Fig. 2 shows that using $\Delta\lambda$=20 nm nearly the same peak at 670 nm is found for both cells in suspension and immobilized in the silica matrix. The peak heights are also very similar, while their intensities become quite different for lower wavelengths. Under these conditions, synchronous-scan fluorescence can be applied to immobilized algal cells for chemical toxicity assessment. In addition, entrapped algal cells can be used as active membranes for biosensors (Naessens et al., 2000).

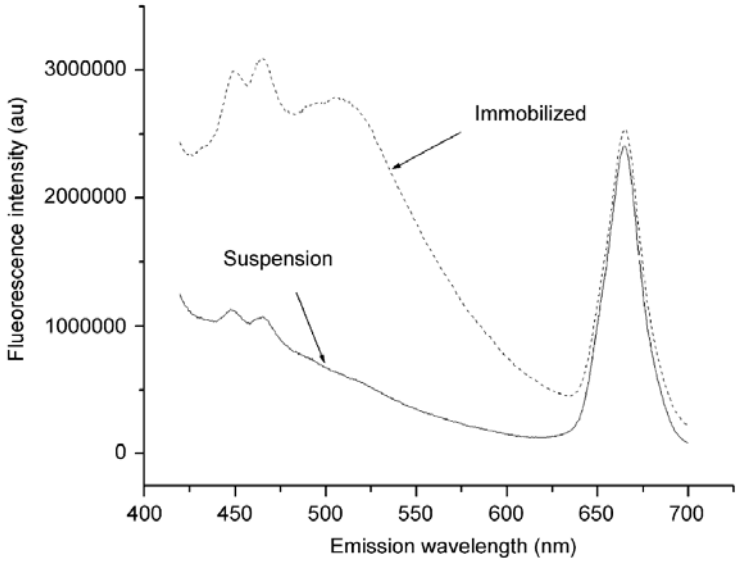

Fig 2: Synchronous fluorescence spectroscopy of "C. vulgaris" cells (35×10⁶ cells/mL) in suspension in water (pH 7) and immobilized in a silica gel using $\Delta\lambda$=20 nm between excitation and emission wavelengths. Fluorescence intensity is expressed in arbitrary unit (au).

Effect of metal concentration

The photosynthetic activity of algal cells is affected by heavy metals at very low concentrations. Fig. 3 shows that synchronous fluorometry is a sensitive method for their toxicity assessment, since the fluorescence quenching efficiency I_0/I increases with metal concentration in the dynamic range. The quenching efficiency is defined as the ratio I_0/I of the fluorescence I_0 in the absence to the fluorescence I in the presence of toxicant. Determination of cadmium is possible within the concentration range 0–150 ppb from a calibration curve. The toxic effect of heavy metal also depends upon the time the metal is in contact with the algal cells. This incubation time from 1 to 7 days increases the fluorescence-quenching efficiency of cadmium as indicated in the figure.

Effect of herbicides concentration

Vegetal cells are the main target of herbicides, and the fluorescence of algal cells is affected by the presence of atrazine, diuron, DNOC and paraquat (Fig. 4). Fluorescence emission at 670 nm with a scan wavelength difference $\Delta\lambda$=20 nm decreases quite regularly with DNOC and paraquat concentrations.

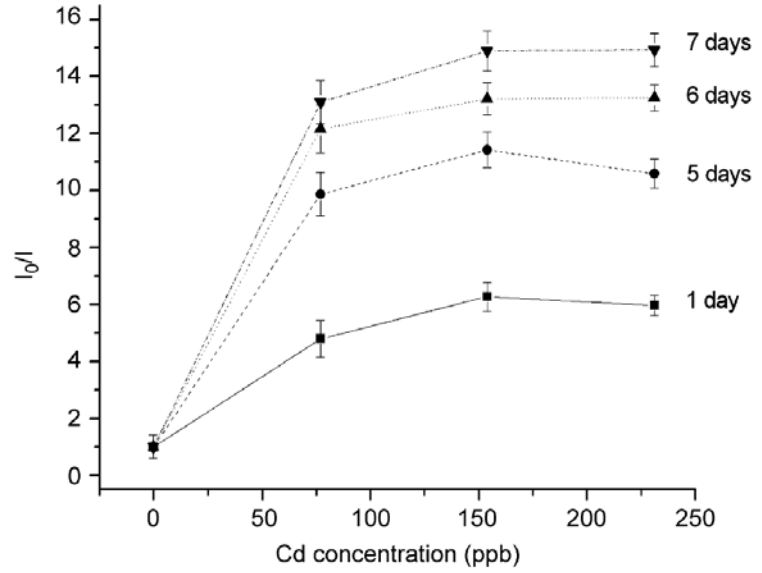

Fig 3: Synchronous fluorescence quenching of immobilized "C. vulgaris" cells (35×10⁶ cells/mL) by cadmium ions as a function of Cd concentration after various periods of incubation time (1–7 days), using Δλ=20 nm and the fluorescence peak at 670 nm.

However, for atrazine and diuron the fluorescence increases when the concentrations are below 1 µg/L and decreases for higher concentrations. This behavior could be ascribed to their specific mode of action on PS I and II.

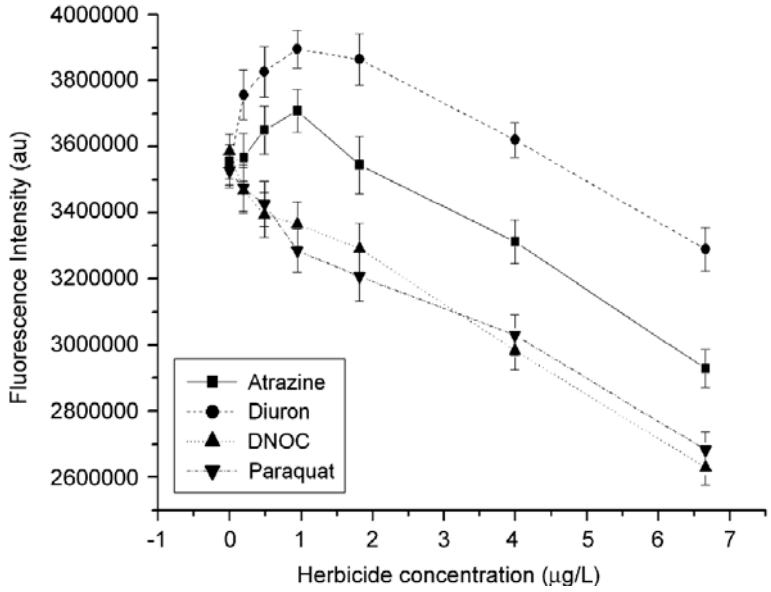

Fig 4:. Synchronous fluorescence of immobilized "C. vulgaris" cells (35×10⁶ cells/mL) as a function of herbicide concentration for various types of herbicides using Δλ=20 nm, and the fluorescence peak at 670 nm after 1 h contact time. Fluorescence intensity is expressed in arbitrary unit (au).

Discussion

In this work, synchronous-scan spectrofluorometry is used to assess the toxic effect of cadmium and herbicides on vegetal cells. It is known that cadmium has a toxic effect on bacteria, algae and fungi (Trevors et al., 1986; Singh and Tewari, 2003; Lin et al., 2007a, b) through its action on enzymes of the metabolic chain (Durrieu and Tran-Minh, 2002).

Fig. 1 shows that it is also possible to use $\Delta\lambda$=90 nm for Cd(II) toxicity testing, as adopted by Liu et al. (2005) for Fe(III) quenching, but the corresponding difference in fluorescence intensity is three times lower than with $\Delta\lambda$=20 nm and consequently reduces the sensitivity of the technique. As indicated in the figure, the most appropriate $\Delta\lambda$ is 20 nm because it corresponds to the greatest reduction in fluorescence intensity in the presence of cadmium compared to all other wavelength differences. This is also the best $\Delta\lambda$ value for toxicity assessment, since it exhibits highest emission peak and good quenching efficiency with regard to the toxicant. For a same quenching efficiency, a greater fluorescence intensity is preferred for its higher signal-to-noise ratio.

In order to apply this technique to immobilized algal cells, a translucent matrix is required to minimize optical interference from the support. This has been obtained by entrapment of algal cells in a porous silica matrix using the sol–gel process (Nguyen-Ngoc and Tran-Minh, 2007a). Silica matrixes are relatively inexpensive to synthesize and have interesting properties, including optical transparency, biocompatibility and chemical inertness. This support also ensures algal suspension homogeneity and is suitable to produce fluorescent membranes for biosensors (Nguyen-Ngoc and Tran-Minh, 2007b).

Toxicity tests employing microalgae to determine the ecotoxic concentration of heavy metals provide important information for predicting the environmental impact of their pollution. Heavy metals are known to have a toxic effect on algal cells by inhibiting their growth (Lin et al., 2007a, b; ISO, 1998; ASTM, 1994). Cell counting, optical density and fluorescence measurements have been used for their growth quantification (Blaise et al., 1997). Synchronous-scan spectrofluorometry provides a new and rapid technique of direct ecotoxicological assessment of heavy metals from fluorescence quenching.

Herbicides play an important role in agricultural practices to control weeds and to avoid their competition with agricultural crops. An undesirable side effect of their use is that they may enter fresh water habitats and destroy nontarget species. Some are highly persistent and can be found in many environments such as soil, sediments and water (Field et al., 2003). Green algae C. vulgaris is very sensitive to herbicides (Ma et al., 2002) and its photosynthesis inhibition can be assessed from synchronous-scan fluorescence technique. A linear relationship is found between fluorescence intensity and concentration of the tested herbicides at concentrations higher than 1 μg/L: this could be applied to their determination in surface water. Since paraquat and DNOC are anti-PSI herbicides, they decrease the algal fluorescence intensity as usually reported (Fai et al., 2007) and confirmed in Fig. 4. Diuron and atrazine, known as PSII inhibitors, increase fluorescence emission as observed in Fig. 4 for low herbicide concentration. This change in fluorescence results from the specific mode of action of herbicides on algal photosystems in contrast to the simple negative physiological effect of growth inhibition. The growth inhibition actually results from various separate effects of toxic compounds on enzymes acting in sequence in the process of photosynthesis inhibition. Synchronous-scan spectrofluorometry experiments with other toxicants will help in providing evidence for discussion on the mode of action of those herbicides and their effect on fluorescence emission.

Conclusion

Synchronous-scan fluorometry can be effectively used to assess the sensitivity of *C. vulgaris* to heavy metals and herbicides. The wavelength difference ($\Delta\lambda$) of 20 nm is optimal for characterization of the quenching effect of these toxic chemicals. On the whole, the higher the toxicant concentration, the greater the quenching efficiency. This result can be used for evaluation of toxic compounds after appropriate calibration. Such quantitative assays may be of interest for analytical purposes, as their concentration gives useful indications on their

degree of photosynthetic inhibition. Atrazine and diuron exhibit a particular behavior which could be ascribed to their specific mode of action on PSII. The emission peak at 670 nm, which is similar for both cells in suspension and immobilized in the silica support, facilitates the determination of toxicants in different media. The technique using algal cells immobilized in a translucent matrix is quite adapted to the construction of algal biosensors. The results are very useful since it demonstrates that synchronous-scan fluorometry could be a very sensitive technique for assessing the effects of toxicants on *C. vulgaris* at very low concentrations.

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