Ecotoxicology (2010) 19:1059–1065
DOI 10.1007/s10646-010-0487-y

TECHNICAL NOTE

Development of a microalgal PAM test method for Cu(II) in waters: comparison of using spectrofluorometry

E. Peña-Vázquez · C. Pérez-Conde · E. Costas · M. C. Moreno-Bondi

Accepted: 14 March 2010 / Published online: 31 March 2010

Abstract Test methods are needed to monitor Cu concentrations in reservoirs and water supplies. *Dictyosphaerium chlorelloides* (Chlorophyta) cells were immobilized in a silicate sol–gel and the toxic effects of Cu(II) were examined using different techniques: fluorescence measurements (using a spectrofluorometer with an optic fiber coupled to a flow cell or a 96-well-plate reader) or by Pulse Amplitude Modulation (PAM) parameters using a portable instrument and the pulse saturation method. Fm′ and qN were the most sensitive indicator parameters when performing Cu analysis in water. *D. chlorelloides* PAM biosensor presented a detection limit of 0.6 mg l^{-1} for Cu(II), within the limits to establish if Cu concentrations exceeded regulatory levels. Moreover, a 1.9 mg Cu l^{-1} (30 μM) resistant strain of the *D. chlorelloides* microalgae was produced in order to obtain more selectivity on the metal determination.

Keywords Biosensor · Algae · *Dictyosphaerium chlorelloides* · PAM · Copper

E. Peña-Vázquez
Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Chemistry, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

C. Pérez-Conde · M. C. Moreno-Bondi
Chemical Optosensors, Laboratory of Applied Photochemistry Group, Department of Organic and Analytical Chemistry, Complutense University of Madrid, Ciudad Universitaria, 28040 Madrid, Spain

E. Costas (✉)
Veterinary Microbiological Control Group, Department of Animal Production (Genetics), Complutense University of Madrid, Ciudad Universitaria, 28040 Madrid, Spain
e-mail: ecostas@vet.ucm.es

Abbreviations

DC	*Dictyosphaerium chlorelloides*
EC_{50}	Effective concentration causing a 50% of variation in the signal (mg l^{-1})
F	Fluorescence yield measured briefly before application of the last saturation pulse; normally measured in the presence of actinic light
Fm	Maximal fluorescence yield of a dark-adapted sample, with all PSII reaction centers fully closed
Fm′	Maximal fluorescence yield reached during last saturation pulse with an illuminated sample
Fo	Minimal fluorescence yield of a dark-adapted sample, with all PSII reaction centers fully open
Fo′	Minimal fluorescence yield of a preilluminated membrane containing the algae; determined briefly after turning off the actinic light and after several seconds of far-red illumination to assure full oxidation of PSII acceptors
Fv	Fv = Fm − Fo
LOD	Limit of detection (mg l^{-1})
PAM	Pulse Amplitude Modulation
PAR	Photosynthetically active radiation
PSII	Photosystem II
qP	Coefficient of photochemical quenching defined by: qP = (Fm′ − F)/(Fm′ − Fo′). It may vary between 0 and 1
qN	Coefficient of nonphotochemical quenching defined by: qN = (Fm − Fm′)/(Fm − Fo′). It may vary between 0 and 1
Y	(=Φ′) Effective quantum yield of photochemical energy conversion at PSII reaction centers calculated according with: Y = (Fm′ − F)/Fm′

Introduction

Since the beginning of the twentieth century, copper sulfate has been the most commonly used algaecide to treat surface waters from lakes, reservoirs and other water supplies. Copper transfers into the food chain, and acts as a primary factor of species selection and have a demonstrable influence on biodiversity in these kinds of environments (Vinot and Pihan 2005). Repetitive algaecide applications in water supplies may lead to the proliferation of copper resistant cyanobacteria, leading to the ineffectiveness of such treatments (García-Villada et al. 2004; Costas and López-Rodas 2006).

Chlorophyll and fluorescence bioassays are rapid and provide new knowledge about the physiological impact of the pollutants, but standardised protocols still need to be established (Ralph et al. 2007). Recently, several papers have studied microalgae response versus metal concentration in solution using PAM: for cadmium (Mallick and Mohn 2003; Miao et al. 2005; Wang and Dei 2006), copper (Juneau et al. 2002; Mallick and Mohn 2003; Bengtson Nash et al. 2005; Miao et al. 2005; Wang and Dei 2006), chromium (Mallick and Mohn 2003; Ait Ali et al. 2008), mercury (Juneau et al. 2001; Kukarskikh et al. 2003), nickel (Mallick and Mohn 2003) and zinc (Mallick and Mohn 2003; Miao et al. 2005). Also, immobilized microalgae could be an inexpensive and appropriate tool to estimate toxicant concentrations (Hameed and Ebrahim 2007).

This study seeks to contribute to the development of a screening method for copper (II) in water. The freshwater green algae *Dictyosphaerium chlorelloides* (*Chlorophyceae*) were encapsulated in a silicate sol–gel. Fluorescence and variation in different PAM parameters were evaluated versus toxic concentration. Aqueous precursors, such as sodium silicate or colloidal silica particles were the preferred option to immobilize bacteria or algae in sol–gels (Bhatia et al. 2000; Coiffier et al. 2001; Chen et al. 2004; Nassif et al. 2003; Nguyen-Ngoc and Tran-Minh 2007; Peña-Vázquez et al. 2009).

A significant problem concerning whole cell biosensors is the lack of selectivity. Thus, some authors have been able to differentiate sensitivity patterns of immobilized algal strains (Podola and Melkonian 2005; Tibuzzi et al. 2007; Chaplen et al. 2007) in order to evaluate toxicant concentrations. The selectivity of our cell biosensors was enhanced by comparing responses of wild-type Cu-sensitive and Cu-resistant-mutants algal cultures, as previously described (Altamirano et al. 2004; Peña-Vázquez et al. 2009).

Methods

Chemicals

In order to generate sol–gels, sodium silicate solution (*purum* $\geq$10% as NaOH, $\geq$27% as SiO_2) (Riedel-de Häen, Seelze, Germany), glycerol (Sigma Chemical Co., St. Louis, MO, USA) and hydrochloric acid (Panreac Barcelona, Spain) were used.

Cu(II)SO_4 pentahydrate, Hg(II) chloride and chromium (VI) oxide (Sigma-Aldrich St. Louis, MO, USA), simazin PESTANAL® (Riedel-de Häen, Sigma-Aldrich Laborchemikalien GMBH, Seelze, Germany). A stock solution of 1000 μg l^{-1} simazine was prepared in DMSO (Scharlau, Barcelona, Spain).

All solutions were prepared using Milli-Q water from a Millipore purification system (Millipore Ibérica, Madrid, Spain). All solutions and BG11-culture media were prepared with autoclaved milli-Q water.

Microalgal culture conditions

Dictyosphaerium chlorelloides microalgae cultures (DC) procured from the UCM algal culture collection were grown axenically in flasks (Greiner) with 20 ml of BG-11 medium (Sigma, St. Louis, MO, USA) at 20°C, under continuous light of 60 μmol $m^{-2}s^{-1}$ over a 400–700 nm waveband. The subsequent cultures (6×10^7 cell ml^{-1}) were encapsulated in silicate sol–gel and spectrofluorometry or PAM parameters were used to test copper concentrations.

In order to improve biosensor selectivity, copper resistant mutants were obtained as previously described (García-Villada et al. 2004) and its resistance was increased using cycles that involved a serial transfer protocol with a ratchet to higher toxicant doses (this was done until 30 μM (1.9 mg l^{-1}) Cu(II) solution was attained). This system maintains large cell populations under selection pressure enabling favorable mutations to be conserved during the selection process (Reboud et al. 2007).

Immobilization of algae: sol–gel encapsulation

Sol–gel was formed by mixing algae solutions with sodium silicate 2 M at pH = 9 and 2.5% glycerol (w/w) to improve biocompatibility and reduce gel shrinkage while drying. Typically, 450 μl of algae solution (6×10^7 cell ml^{-1}) was mixed with 37.5 μl of water and 12.5 mg of glycerol. Later, 60 μl of 2 M HCl and 50 μl of 2 M sodium silicate were added. The mixture was shaken vigorously and 30 μl was deposited on glass supports. Similarly, 10 μl

was deposited in each of the wells of 96-well glass bottom plates for Micromax measurements. After 40 min, membranes were stored in the culture medium BG-11 and kept in darkness at 4°C.

Fluorescence measurements

Spectrofluorometry (fiber optic coupled to a flow cell)

Fluorescence measurements were carried out using a Spex Fluorolog 2 spectrofluorometer (Jovin-Ivon, Longjumeau Cedex, France) at room temperature. Excitation was carried out at 467 nm and emitted light was collected at 699 nm. Excitation and emission slits were 25 nm. An emission 695 nm band pass filter was included in the fiber-optic interface of the spectrofluorometer to diminish light dispersion through the optic fiber. Glass supports including the algae membranes were placed at the tip of a bifurcated fiber-optic cable (1 m long) in a flow cell chamber for measurements. The algae remained in darkness for 15 min prior to irradiation while the sample was streaming through the flow cell. The membrane was irradiated for 5 min to achieve signal stabilization. Photosynthetic response was monitored and the last fluorescence value was recorded (integration time: 1 s).

Spectrofluorometry (fiber optic coupled to a 96-well-plate reader)

A Micromax 384 plate reader, capable of connecting to the SPEX Fluorolog spectrofluorometer was used to carry out fast measurements of fluorescence signals of algae in contact with various copper concentration solutions. The Micromax 384 was capable of scanning 96-Well 0.72 mm Low Glass Bottom Plates (MatriCal Inc., Washington, USA) with algae immobilized in sol–gels at the bottom of the wells. An emission 590 nm band pass filter was included in the fiber-optic interface of the spectrofluorometer to diminish light dispersion. Fluorescence was collected at 684.7 nm. Integration time was 1 s resulting in 96 s as the total determination time for the whole plate. Additionally, a short time was needed to move the plate through stationary fiber optic.

Pulse amplitude modulation

A portable PAM-2000 chlorophyll fluorometer was used to investigate photosynthesis and Cu response (Heinz Walz GmbH, Effeltrich, Germany). Chlorophyll fluorescence was induced by μs-pulses of measuring light derived from light-emitting diodes (LEDs) and the yield of pulse modulated fluorescence was selectively measured (Pulse Amplitude Modulation, PAM). Saturating pulses of light were applied to induce a transient block of photochemical energy conversion and a maximum yield of chlorophyll fluorescence. Fluorescence yield was monitored using multiple parameters and the raw data were evaluated by the DA-2000 data acquisition system in a portable PC. Glass supports containing algae membranes were placed at the tip of a PAM-2000 fiber-optic cable in a flow cell for the measurements. Parameters (Fo′, Fm′, Y, qP, qN) were calculated after allowing contact time between solution and membrane (usually 75 min) and 3 min of irradiation using measuring light (3 μs pulses, maximum emission at 655 nm; intensity level 10) at 600 Hz modulation frequency. Those parameters were measured in conjunction with a series of 400 ms saturation pulses, usually after five saturation pulses separated by 20 s (saturation pulse light source maximum emission <710 nm, intensity 8). When a saturation pulse is applied, the measuring light frequency is switched to 20 kHz with a measurable actinic effect (overall intensity more than 10 μmol quanta m^{-2} s^{-1} PAR). Three seconds after the termination of a saturation pulse, far-red light was applied for 3 s (maximum emission 735 nm, intensity 6, max. 15 W m^{-2}) and Fo′ was calculated. Next, qP and pN were calculated on the basis of Fo′.

Statistics

Obtained data were exported to a Sigma Plot 9.0 datasheet (Systat Software Inc., Richmond, CA, USA), which includes a powerful non-linear curve fitter to treat experimental results and obtain dose–response curves.

Results and discussion

Spectrofluorimetry measurements
(fiber optic coupled to a flow cell)

Response of biosensor membranes were stabilized by passing BG-11 through the flow cell, for not less than 1 h and up to 2 h, and performing cycles of darkness/irradiation. An experiment was performed for three different silicate membranes containing the immobilized algae. Fluorescence intensity response (I) at 5 min irradiation was represented vs. logarithm of copper concentration. It was observed that fluorescence values (average ± SD; $n = 3$ membranes from different algae cultures) increased with doses from 0.1 mg l^{-1} to 10 mg l^{-1} of copper. Later, there was a sharp decrease when copper concentration was approximately 15–20 mg l^{-1}.These types of measurements would not be useful for copper determination, since they would not be able to distinguish the copper concentration corresponding to a fluorescence signal value from a calibration curve.

Spectrofluorimetry measurements (fiber optic coupled to a 96-well plate reader)

Calibrations were performed in quadruplicate for wild-type DC and 1.9 mg l^{-1} Cu(II) resistant DC mutants. Measurements were performed using 10 µl of sol–gel containing algae immobilized was placed in each well of the plate. 200 µl of solution containing copper was added to each well. Proceeding, variations of fluorescence signal with concentration and time was studied. It could be observed that to obtain a decrease in the signal of more than 10% for 2.5 mg Cu l^{-1} added, algae need to be in contact with the solution for 48 h. A value of 60% of the initial signal corresponded to a Cu concentration of 30 mg l^{-1}. Similarly, relatively long time intervals are needed to observe differences in the response for wild-type algae and resistant algae: 450 min for a 70 mg l^{-1} solution or 48 h for a 10 mg l^{-1} copper solution. Therefore, this method is time consuming and not sensitive.

PAM measurements

Variation of PAM parameters with time

The maximum potential fluorescence yield is quenched by two different types of competing de-excitation processes: photochemical energy conversion at the PSII centers (photochemical quenching) and non-photochemical loss of excitation energy at the antenna and reaction center levels, usually by heat dissipation (non-photochemical quenching). These two options can be distinguished by PAM using the "saturation pulse method".

Calibrations were performed using different membranes, leaving algae in contact with copper solutions in the flow cell for selected times (3, 10, 30, 60 or 75 min) before measuring light irradiation time and taking measurements with the saturation pulse. Irradiation time 3 min for all samples and sample flow was 1.5 ml min^{-1}. Figure 1 shows variation for Fo′, Fm′, yield, qP and qN. Parameter values for a BG-11 solution represent a reference signal value = 100 except for qN. The highest variation was observed for Fm′ and qN. qN was the parameter which reflected the greatest variation with copper concentration while 75 min was sufficient time to obtain a useful signal to develop a method for copper analysis in drinking waters.

Calibration (qN)

Figure 2 shows signals for increasing copper concentrations. qN is represented vs. logarithm of Cu concentration. Experimental points were adjusted to a four parameters logistic curve (Eq. 1)

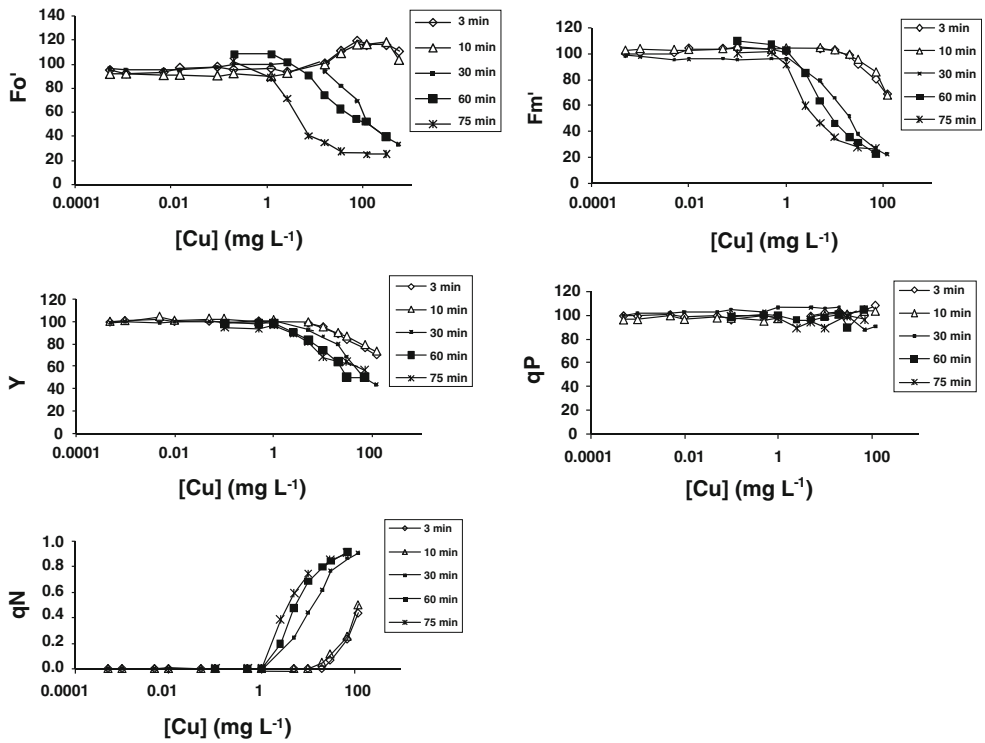

Fig. 1 PAM: variation of Fo′, Fm′, yield, qP and qN with copper concentration and contact time with the algae

$$I = \min + \frac{\max - \min}{\left(1 + {}^{[Cu]}/_{EC50}\right)^{Hillslope}} \quad (1)$$

where "min" is the lowest value of where "min" is the lowest value of the calibration curve and "max" the highest value. qN was calculated after 5 saturation pulses as a higher sensitivity was obtained. A typical response curve ($r > 0{,}995$) for sensitive wild-type DC membranes was also be observed. Average and standard deviations are shown for three different membranes corresponding to three different DC algae cultures. Dose–response calibrations were performed, and limits of detection were calculated: $I_{LOD} = \min + 0.1(\max–\min)$. Limit of detection was 0.65 ppm. The dynamic range from calibration ranges from 1.1 to 8.4 mg l^{-1}, and the EC_{50} was 3.1 mg l^{-1}.

The LOD obtained was less than the limit established for water for human consumption. European and Spanish legislation has established maximum Cu concentrations in water used for human consumption as 2 mg l^{-1} (Council Directive 98/83/EC 1998; REAL DECRETO 140/2003 2003) or 1 mg l^{-1} for natural mineral water (REAL DECRETO 1744/2003 2003). Copper response for resistant DC membranes are also included in Fig. 2. As can be observed, wild-type DC algae and resistant algae have different responses to copper solutions corresponding to 1 and 2.5 mg l^{-1}. These findings (low detection limit and selectivity) imply that a dual strain Cu biosensor could be applied as a screening method to test for copper presence in water in accordance with legislation, or at least, to separate groups of substances affecting the same targets in photosystem II.

Study of interferences

In order to test how interferences affect a Cu sensor, PAM responses for 2 mg l^{-1} solutions were compared with those prepared with 2 mg l^{-1} of copper plus another toxicant. The studied toxicant concentrations were as follows: 50 μg l^{-1} of Cr, 1.0 μg l^{-1} of Hg, and 0.1 μg l^{-1} of simazin (limit values established by Spanish legislation: REAL DECRETO 140/2003 2003). It was observed that variation in the obtained results (qN) was similar to those ones obtained only for copper while using different membranes and cultures.

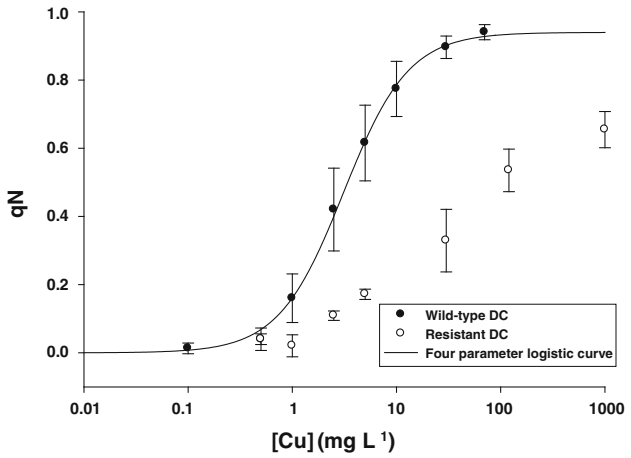

Fig. 2 PAM: wild-type sensitive DC response (logistic response model fit) vs. resistant DC response

Feasibility of the PAM test method as a tool for biomonitoring

Study of chlorophyll a fluorescence in ecotoxicology involves applying Chlorophyll a parameters as endpoints to assess the magnitude of toxic impact. This can be done using PAM. In this way, it can be evaluate the mechanism of action of the toxicant on the photosynthetic electron transport chain. However, photosynthetic impact of metals is controversial. The majority of authors in this topic have stated that the target site of Cu impact on PSII is at its oxidizing side, although other possible impact sites could be the PSII reaction center, the Pheo-Q_A-Fe^{2+}, within the Hill reaction and during water splitting. Moreover, light intensity irradiation plays an active role as well (Ralph et al. 2007).

Several papers have compared the sensitivity of different endpoints using PAM. Φ′(=Y) and q_N seem to be the most sensitive fluorescence parameters for copper bioassays (Juneau et al. 2002). EC_{50} values were lower than in this study (11–34 μg l^{-1} for *Chlamydomonas reinhardtii*; 25–50 μg l^{-1} for *Selenastrum capricornutum*, for times of exposure ranging from 5 to 96 h). However, *Chlorella vulgaris* did not show response even at 100 μg l^{-1}. In a later work (Miao et al. 2005), the authors observed that PAM parameters (maximum and operational quantum yield) and specific growth rate were comparable as trace metal toxicity biomarkers using four marine phytoplankton species. They compared the cellular metal concentration at NOEC (no-observed-effect-concentration). Their sensitivities as biomarkers for trace metal toxicity (Cd, Cu, Zn) showed EC_{50} values even lower than in the first work. Over the 96 h period, the cyanobacteria *Synechococcus* sp. was the most sensitive specie studied because of its higher cell surface to volume ratio. In both studies, concentrations of toxicant up to 100 or 7 μg l^{-1} were studied with values being much lower than those reflected in legislation.

In other studies, organisms showed quicker response, e.g. *Chlorella vulgaris* (IC_{10} of 51 μg l^{-1}, showing inhibition together with a large decrease in fluorescence yield within 25 min) (Bengtson et al. 2005), or *Scenedesmus obliquus* at higher concentration values (Fm, Fv/2 and Fo/Fv showed variations after 1 h of treatment; EC_{50} = 200 μmol l^{-1} = 12.7 mg l^{-1}) (Mallick and Mohn 2003).

In comparison to each of the aforementioned values, the LOD, range of concentrations evaluated and relatively short response time (75 min) found in the present work suggests that field applications are possible using an immobilized algae. Further studies are ongoing in order to produce more sensitive and more resistant algae to copper and to develop a dual strain (sensitive and resistant) algae biosensor for copper in water. Further research is needed in the area to improve reproducibility as well as study the influence of several parameters, such as selection of indicative PAM parameters (Juneau et al. 2005), culture protocols, measurement time after preparing the sol–gel or light history of the samples.

Conclusions

After immobilisation of *Dictyosphaerium chlorelloides* microalgae in a silicate matrix, its response to copper was studied using spectrofluorimetry and PAM. Several conclusions can be made from this study. Firstly the PAM saturation pulse method was the preferred option for carriying out Cu analysis in water. Secondly Fm′ and especially qN were the most sensitive PAM parameters for performing Cu field screening in water. Thirdly, the *Dictyosphaerium chlorelloides* PAM biosensor presented a detection limit of 0.6 mg l^{-1} for Cu(II) detection, which was a low enough value to be able to determine if Cu concentrations exceeded regulatory levels (2 mg l^{-1}). A lastly, the 1.9 mg Cu l^{-1} (30 μM) resistant strain of the microalgae was used in order to assure selectivity on Cu determination with no measurable response at the regulatory level.

Acknowledgements This study received financial support from the Comunidad Autónoma de Madrid (Project S0505/AMB374), the ESF, the ERDF. Elena Peña Vázquez acknowledges the Isidro Parga Pondal Program (Xunta de Galicia) for her research grant in the Complutense University.

References

Ait Ali N, Juneau P, Didur O, Perreault F, Popovic R (2008) Effect of dichromate on photosystem II activity in xanthophylls-deficient mutants of *Chlamydomonas reinhardtii*. Photosynth Res 95: 45–53

Altamirano M, García-Villada L, Agrelo M, Sánchez-Martín L, Martín-Otero L, Flores-Moya A, Rico M, López-Rodas V, Costas E (2004) A novel approach to improve specificity of algal biosensors using wild-type and resistant mutants: an application to detect TNT. Biosens Bioelectron 19:1319–1323

Bengtson Nash SM, Quayle PA, Schreiber U, Muller JF (2005) The selection of a model microalgal species as biomaterial for a novel aquatic phytotoxicity assay. Aquat Toxicol 72:315–326

Bhatia RP, Brinker CJ, Gupta AK, Singh AK (2000) Aqueous solgel process for protein encapsulation. Chem Mater 12:2434–2441

Chaplen FWR, Vissvesvaran G, Henry EC, Jovanovic GN (2007) Improvement of bioactive compound classification through integration of orthogonal cell-based biosensing methods. Sensors 7:38–51

Chen J, Xu Y, Xin J, Li S, Xia C, Cui J (2004) Efficient immobilization of whole cells of *Methylomonas* sp. strain GYJ3 by sol-gel entrapment. J Mol Catal B-Enzym 30:167–172

Coiffier A, Coradin T, Roux C, Bouvet OMM, Livage J (2001) Sol-gel encapsulation of bacteria: a comparison between alkoxide and aqueous routes. J Mater Chem 11:2039–2044

Costas E, López-Rodas V (2006) Copper sulphate and DCMU-herbicide treatments increase asymmetry between sister cells in the toxic cyanobacteria *Microcystis aeruginosa*: Implications for detecting environmental stress. Water Res 40:2447–2451

Council Directive 98/83/EC of 3 November (1998) On the quality of water intended for human consumption. Official Journal of the European Communities L 330:32–54

García-Villada L, Rico M, Altamirano M, Sánchez-Martín L, López-Rodas V, Costas E (2004) Occurrence of copper resistant mutants in the toxic cyanobacteria *Mycrocistis aeruginosa*: characterisation and future implications in the use of copper sulphate as algaecide. Water Res 38:2207–2213

Hameed MSA, Ebrahim OH (2007) Biotechnological potential uses of immobilized algae. J Agric Biol 9:183–192

Juneau P, Dewez D, Matsui S, Kim SG, Popovic R (2001) Evaluation of different algae species sensitivity to mercury and to metolachlor by PAM-fluorometry. Chemosphere 45:589–598

Juneau P, El Berdey A, Popovic R (2002) PAM fluorometry in the determination of the sensitivity of *Chlorella vulgaris, Selenastrum capricornutum*, and *Chlamydomonas reinhardtii* to copper. Arch Environ Contamin Toxicol 42:155–164

Juneau P, Green BR, Harrison PJ (2005) Simulation of Pulse-Amplitude-Modulated (PAM) fluorescence: limitations of some PAM-parameters in studying environmental stress effects. Photosynthetica 43:75–83

Kukarskikh GP, Graevskaya EE, Krendeleva TE, Timofeev KN, Rubin AB (2003) Effect of methylmercury on the primary photosynthetic activity of green microalgae *Chlamydomonas reinhardtii*. Biofizika 48(5):853–859

Mallick N, Mohn FH (2003) Use of chlorophyll fluorescence in metal-stress research: a case study with the green microalga *Scenedesmus*. Ecotoxicol Environ Saf 55(1):64–69

Miao AJ, Wang WX, Juneau P (2005) Comparison of Cd, Cu, and Zn toxic effects on four marine phytoplankton by pulse-amplitude-modulated fluorometry. Environ Toxicol Chem 24: 2603–2611

Nassif N, Roux C, Coradin T, Rager MN, Bouvet OMM, Livage J (2003) A sol-gel matrix to preserve the viability of encapsulated bacteria. J Mater Chem 13:203–208

Nguyen-Ngoc H, Tran-Minh C (2007) Fluorescent biosensor using whole cells in an inorganic translucent matrix. Anal Chim Acta 583:161–165

Peña-Vázquez E, Maneiro E, Pérez-Conde C, Moreno-Bondi MC, Costas E (2009) Microalgae fiber optic biosensors for herbicide monitoring using sol-gel technology. Biosens Bioelectron 24: 3538–3543

Podola B, Melkonian M (2005) Selective real-time herbicide monitoring by an array chip biosensor employing diverse microalgae. J Appl Phycol 17:261–271

Ralph PJ, Smith RA, Macinnis-NG CMO, Seery CR (2007) Use of fluorescence-based ecotoxicological bioassays in monitoring toxicants and pollution in aquatic systems; review. Toxicol Environ Chem 89:589–607

REAL DECRETO 140/2003 de 7 de febrero (2003) Por el que se establecen los criterios sanitarios de la calidad del agua de consumo humano. BOE 45:7228–7245

REAL DECRETO 1744/2003 de 19 de diciembre (2003) por el que se modifica el Real Decreto 1074/2002, de 18 de octubre, por el que se regula el proceso de elaboración, circulación y comercio de aguas de bebida envasadas. BOE 312:46524–46529

Reboud X, Majerus N, Gasquez J, Powles S (2007) *Chlamydomonas reinhardtii* as a model system for pro-active herbicide resistance evolution research. Biol J Linn Soc 91:257–266

Tibuzzi A, Rea G, Pezzotti G, Esposito D, Johanningmeier U (2007) A new miniaturized multiarray biosensor system for fluorescence detection. J Condens Matter 19:1–12

Vinot I, Pihan JC (2005) Circulation of copper in the biotic compartments of a freshwater reservoir. Environ Pollut 133: 169–182

Wang WX, Dei RCH (2006) Metal stoichiometry in predicting Cd and Cu toxicity to a freshwater green alga *Chlamydomonas reinhardtii*. Environ Pollut 142:303–312