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Suitability of cytotoxicity endpoints and test microalgal species to disclose the toxic effect of common aquatic pollutants

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ABSTRACT

Pulse discharges of chemicals to aquatic environments may lead to high concentrations of them in surface waters for short periods of time, but enough to induce toxic effects on aquatic organisms; however, no many methods allow an early warning of toxicity of these agents. Acute effects of one representative chemical from each of three of the main groups of aquatic pollutants (pesticides, metals and pharmaceuticals) are studied on two green microalgal species (*Chlamydomonas moewusii* and *Chlorella vulgaris*). Flow cytometry protocols were used to detect the potential application of chlorophyll *a* fluorescent emission, cell viability, metabolic activity and membrane potential as cytotoxicity endpoints, besides an epifluorescence microscopy protocol for comet assay to detect genotoxicity level of cells. Obtained results confirm the suitability of them for the prospective assessment of the potential cytotoxicity of these aquatic pollutants. The two microalgal species analysed could be used as indicators in toxicity bioassays, being *C. moewusii* more sensitive than *C. vulgaris*. Among cell parameters assayed, the metabolic activity and the primary DNA damage stood out as sensitive cytotoxicity endpoints.

1. Introduction

The many ecological disturbances in aquatic ecosystems linked to anthropogenic pressures are well documented (Amado et al., 2006; Corrêa et al., 2009; Moreira-Santos et al., 2004). The widespread use of pesticides has led to these substances be ubiquitous in aquatic environments (Eullaffroy and Vernet, 2003; Kumari et al., 2007). Metals are also major pollutants of aquatic ecosystems, mainly due to disposal of industrial effluents or mining activity (Franklin et al., 2000; Pan and Wang, 2012). Pharmaceutically active substances have been recognised as an important environmental problem (Halling-Sorensen et al., 1998; Pomati et al., 2004).

The increasing concern about environmental pollution has led to the development of sensitive analytical methods to detect toxicity in water (Camacho-Muñoz et al., 2010; Infante et al., 2008; Kolpin et al., 1998; Núñez et al., 2002), but most of these techniques are expensive, time-consuming and cannot provide information of ecological relevance. Therefore, the development of convenient methods or parameters for the assessment of pollutant toxicity on aquatic organisms has become a major goal in ecotoxicological research (Lam and Gray, 2003).

Microalgae have been frequently used in ecotoxicological screening of contaminated water, and also as test microorganisms for *in vitro* toxicity bioassays. Several parameters can be measured to assess the effects of toxicants on microalgae, being growth and photosynthetic activity the most commonly monitored (Cleuvers, 2003; Shabana et al., 2001; Yang et al., 2002).

Previous studies of the toxic effects of different herbicides on microalgal physiology (Prado et al., 2011, 2012a, 2012b; Rioboo et al., 2009) demonstrate that flow cytometric analysis of different microalgal cell responses can be an alternative to standard algal population-based endpoints, since they allow a rapid measurement of functional responses of individual cells to stress, avoiding loss of information due to obtain average values from the simultaneous analysis of elevated cell numbers. Furthermore, measuring primary DNA damage on microalgae by means of the comet assay is a sensitive genotoxicity biomarker (Prado et al., 2009). Then, the main objective of the present study is to prove the suitability of these cytomic techniques applied on microalgal bioassays to evaluate the potential acute toxicity of other chemical pollutants in freshwater environments. The effects of a representative chemical from each of three main groups of aquatic pollutants (pesticides, metals and pharmaceuticals) are studied on two freshwater green microalgae (*Chlamydomonas moewusii* and *Chlorella vulgaris*) by means of cytometric assays (chlorophyll *a* autofluorescence, cell viability, metabolic activity, cytoplasmic membrane potential) and the comet assay. Also, the different response level of the two microalgal species used will be compared

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to check which of them is more suitable for its use in toxicity bioassays.

2. Materials and methods

2.1. Microalgal cultures and chemical treatments

C. moewusii Gerloff (*Chlamydomonadaceae*) (CCAP 11/5B) and *C. vulgaris* Beijerinck (*Chlorellaceae*) (CCAP 211/11B) were cultured in sterile inorganic Bristol medium (Brown et al., 1967).

All tests were carried out in 100 ml Pyrex glass bottles containing 50 ml of culture, in an incubator under controlled conditions according to that established for stock cultures: 18 ± 1 °C, illuminated with a photon flux of 70 μmol m^{-2} s^{-1} under a dark: light cycle of 12:12 h, obtaining synchronized cultures (Altenburger, 2007). Microalgal cells in early exponential growth phase were used as inoculum for the assays. Initial cell density was adjusted to 1.5 μg chlorophyll per ml for both species assayed.

Fungicide imazalil stock solution was prepared by dissolving granulated pure pesticide in methanol, while copper sulphate and ibuprofen-Na stock solutions were prepared in distilled water. Pollutant concentrations were selected to observe their potential cytotoxic effects on cultured microalgal cells, not depending of their environmental relevance. Solutions were prepared on day prior to use in each experiment, and then diluted in the culture media to reach the final tested concentrations: ranged from 1.5 to 24 mg/L for cultures exposed to imazalil, from 1 to 30 mg/L for cultures exposed to copper; and from 25 to 180 mg/L for cultures exposed to ibuprofen-Na. To achieve these nominal concentrations of all pollutants, stock solutions volume added to the microalgal cultures never exceed 1% of final volume. Cultures without tested chemicals were included as controls; in the case of imazalil, no significant effects of the solvent were observed. All cultures were carried out in triplicate, and different analyses were done after 3 and 24 hours of exposure to the tested chemical.

2.2. Flow cytometric analysis

Flow cytometric analysis of microalgal cells were performed in a Coulter Epics XL4 flow cytometer (Beckman Coulter Inc.) equipped with an argon-ion excitation laser (488 nm), detectors of forward (FS) and side (SS) light scatter and four fluorescence detectors corresponding to different wavelength intervals: 505–550 nm (FL1), 550–600 nm (FL2), 600-645 nm (FL3) and $>$ 645 nm (FL4). Forward scatter and red chlorophyll fluorescence histograms were used to characterize the microalgal population, setting gating levels in order to exclude non-microalgal particles. For each cytometric parameter investigated, at least 10^4 gated cells were analysed per sample and fluorescence measurements were obtained in a logarithmic scale. Data were collected using listmode files and statistically analysed using the EXPO32 ADC software (Beckman Coulter Inc.). Aliquots of microalgal cultures were re-suspended in phosphate buffered saline solution (PBS, pH 7.4) and analysed by FCM to study the potential alterations in the red autofluorescence (FL4), related to the chlorophyll *a* fluorescence emission, an inherent cell property on microalgae.

Besides this inherent cell property, FCM was used in combination with different fluorochromes to analyse other physiological cell parameters. Cell suspensions (2×10^5 cells/mL for *C. moewusii* and 1×10^6 cells/mL for *C. vulgaris*) were incubated with the appropriate fluorochrome at room temperature and darkness for the necessary time. The lowest fluorochrome concentration and the shortest incubation time were chosen in order to obtain significant and stable staining of cells without toxicity being developed.

Cell viability was analysed by incubation of cell suspension with propidium iodide (PI) at a final concentration of 4 μM, for both microalgal species; this fluorochrome allows discriminating between live non-fluorescent cells, with an intact cellular membrane, and non-viable fluorescent cells, with permeability problems at the membrane level (Prado et al., 2009; Rioboo et al., 2009), being the orange fluorescent emission of this compound collected in the FL3 channel indicated above.

Cytoplasmic membrane potential was monitored using a slow-response potentiometric probe, the bis-(1,3-dibutylbarbituric acid) trimethine oxonol ($DiBAC_4(3)$); final concentration and incubation time: 1 μM 10 min for *C. moewusii*, and 5 μM 30 min for *C. vulgaris*. Cytoplasmic membrane depolarisation will be reflected in an increased intracellular anionic dye concentration, i.e. by accumulation of dye in the cells, whilst decreased accumulation will reflect hyperpolarisation (Prado et al., 2012b). $DiBAC_4(3)$ green fluorescent emission was collected in the FL1 channel indicated above.

Metabolic activity, or cell vitality, was assessed using a fluorescein diacetate (FDA)-based cell esterase activity assay, a sensitive and rapid technique to assess phytoplankton metabolic activity (Jochem, 1999; Prado et al., 2009). A kinetic approach to the FDA assay (*in fluxo* analysis) was used in this work, recording the increase of the FDA-dependent fluorescence after FDA addition in the FL1 channel (final concentration: 0.2 μM for *C. moewusii* and 0.6 μM for *C. vulgaris*), depending on time, which allowed calculating the fluorescence generation rates in arbitrary units per minute (Prado et al., 2012b).

2.3. Comet assay

The alkaline single-cell gel electrophoresis or comet assay was applied to detect the primary DNA damage potentially induced by the exposure of microalgae to the tested chemicals. The comet assay protocol used is a modification of the original protocol (Singh et al., 1988) adapted to planktonic algae by Erbes et al., (1997), with an additional modification that is the DNA staining with SYBR Green I (Prado et al., 2009).

Two replicate slides were prepared for each treatment culture and negative control; furthermore, a positive control was also included (exposure of microalgal cultures to hydrogen peroxide at a final concentration of 0.6 mM). Slides were observed using an epifluorescence microscope Nikon Eclipse E400, with blue light as excitation light. Results are expressed as the percentage of comets *vs*. the total amount of nuclei analysed (at least 50 randomly chosen nuclei per slide).

2.4. Data analysis

Mean and standard deviation (S.D.) values were calculated for each treatment from two independent replicate experiments. To determine significant differences among test concentrations, data were statistically analysed by overall one-way analysis of variance (ANOVA) using SPSS 16.0 software. A p-value < 0.05 was considered statistically significant. When significant differences were observed, means were compared using the multiple-range Duncan test.

3. Results and discussion

3.1. Chlorophyll a fluorescence

The chlorosis state consists of a low level of residual photosynthesis, in which both photosystems gradually lose their activity, and chlorophyll are degraded to reach a residual content. The exposure of *C. moewusii* to any of the tested chemicals provoked an early effect on the natural cell autofluorescence, with the appearance of chlorotic cells (without chlorophyll fluorescent