An in situ bioassay for freshwater environments with the microalga *Pseudokirchneriella subcapitata*

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Abstract

This study was designed to evaluate the suitability of an in situ microalgal bioassay with *Pseudokirchneriella subcapitata* to be used in freshwater environments. The assay potential was investigated by deploying it in a system impacted by acid mine drainage. Water samples were collected to perform a laboratory assay also. *P. subcapitata* was viewed to be a good option for the in situ assay because it grew well and according to control acceptability criteria when immobilized in calcium alginate beads. A reduction in algal growth was apparent at both impacted sites demonstrating assay sensitivity: the site closest to the effluent discharge was clearly impacted and the one further downstream appeared to be moderately impacted. Results from the laboratory assay, designed to distinguish effects of nutrient differences across sites from those due to the effluent, confirmed the in situ responses. Results are discussed in light of the significance of combining information from different assessment tools, namely in situ and laboratory assays, as well as water-quality parameters, particularly at sites that are moderately impacted.
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1. Introduction

Relying solely on laboratory-derived toxicity estimates to determine the impact of contaminants on ecosystems raises concerns about the resultant environmental decisions, since laboratory bioassays are generally conducted under unrealistic conditions. On the other hand, in situ testing offers the possibility of assessing contaminant effects under fluctuating natural environmental conditions, integrating site-specific physical, chemical and biological processes with a minimum of manipulation (Chappie and Burton, 2000). In situ testing is thus acknowledged as a means for exploring the problem of ecological relevance in ecological risk assessment. This recognition has prompted, in recent years, a huge increase in the number of research studies on the development of in situ assay methodologies for various groups of aquatic organisms, including phytoplankton (Crumpton and Wetzel, 1982; Faafeng et al., 1994; Culp et al., 2000; Moreira dos Santos et al., 2002), clams (Ringwood and Keppler, 2002), cladocerans (Pereira et al., 1999, 2000; McWilliam and Baird, 2002), amphipods (Chappie and Burton, 1997; DeWitt et al., 1999), chironomids (Chappie and Burton, 1997; Sibley et al., 1999; Castro et al., 2003), and fish (Jones and Sloan, 1989).

In situ bioassays, however, present some drawbacks associated with confounding factors which complicate the establishment of a causal link between exposure and effects (Chappie and Burton, 2000; Culp et al., 2000). Confounding factors are related mainly to organism transportation and caging, toxicant exposure regime variability (DeWitt et al., 1999; Sibley et al., 1999; Chappie and Burton, 2000; Culp et al., 2000; Moreira dos Santos et al., 2002; Castro et al., 2003) and environmental conditions (Chappie and Burton, 1997; Maltby et al., 2002; Ringwood and Keppler, 2002; Castro et al., 2003). Therefore, to more accurately assess ecosystem quality, in situ assays should ideally be used as part of integrative toxicity assessments, in combination with other ecotoxicological tools such as physico-chemical parameters, biological monitoring surveys, and laboratory assays (Clements and Kiffney, 1994; Maltby and Crane, 1994; Culp et al., 2000; Cherry et al., 2001).

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In contrast to fish and invertebrates, the number of studies concerned with microalgae in situ assays is rather limited, even though algal testing has a clear justification with regard to environmental protection. Algae play a major ecological role in most ecosystems (Lewis, 1995) and have been shown to be relatively sensitive to toxicants in general (Nyholm and Källqvist, 1989; Geis et al., 2000; Weyers et al., 2000). Moreover, algal laboratory assays are commonly applied in environmental studies for various purposes (Nyholm and Källqvist, 1989; Lewis, 1990; Weyers et al., 2000). To compensate for this gap, an in situ microalgal assay using the diatom *Phaeodactylum tricornutum* immobilized in alginate beads has been recently developed, and its potential to assess contaminant effects in estuarine environments demonstrated (Moreira dos Santos et al., 2002). The immobilization of microalgae in beads of calcium alginate is an attractive alternative to other in situ methodologies developed previously, namely dialysis bags and point estimates of algal growth potential (Crumpton and Wetzel, 1982; Davis et al., 1988). It greatly simplifies the handling of the algae and the recovery of cells at the end of the assay, and avoids the loss of algae by grazing, water flow, sedimentation, or biofouling during testing (Bozeman et al., 1989; Faafeng et al., 1994).

The main aim of this study was to adapt and evaluate the in situ microalgal bioassay chambers and procedures, initially developed for estuarine environments, to be used in freshwater systems. For this, a freshwater microalgal species was selected and an in situ assay was performed in a freshwater system impacted by acid drainage from a cupric-pyrite mine. Another objective of this study was to integrate information from the in situ assay and a laboratory assay performed following standard guidelines, in an attempt to more comprehensively interpret the responses obtained from the in situ assay, and at the same time to evaluate the role of laboratory assessments in ecological risk assessment studies.

2. Materials and methods

2.1. Test organism

The microalga *Pseudokirchneriella subcapitata* (Koršhikov) Hindak (previously named *Raphidocelis subcapitata* Koršhikov and *Selenastrum capricornutum* Printz), was the species selected to perform the in situ bioassay for various reasons. It is a species easily available (from culture collections) and maintained in the laboratory under reproducible culture conditions (Nalewajko and Olaveson, 1998). Essentially, *P. subcapitata* is among the most widely used and recommended species for freshwater toxicity testing, for which

standard guidelines have already been established (OECD, 1984; Environment Canada, 1992; USEPA, 1994) and are currently endorsed for regulatory purposes (Lewis, 1990; Weyers et al., 2000). Moreover, a large database is already available on the responses of *P. subcapitata* to a variety of contaminants and its relative sensitivity compared to other test organisms (Radix et al., 2000; Weyers et al., 2000).

P. subcapitata (strain No. WW 15-2521) was acquired from the Carolina Biological Supply Company (Burlington, NC) and maintained in nonaxenic batch cultures, in cotton-stoppered 250-mL Erlenmeyer flasks, filled with 100 mL of Woods Hole MBL growth medium (Stein, 1973). Cultures were incubated in a temperature-controlled room ($20 \pm 1°C$) under continuous cool-white fluorescent illumination (lateral disposition; 7000 lx). New cultures were started from an inoculum obtained by harvesting algae while still in the exponential growth phase (between Days 5 and 7). Cultures were kept under these standardized conditions for 4 months prior to experiments.

2.2. Cell immobilization

The method used for the immobilization of algal cells in beads of calcium alginate was based on that described by Bozeman et al. (1989) and Moreira dos Santos et al. (2002). A 1.3% (w/v) solution of sodium alginate (Sigma Chemical, A-7128, Steinheim, Germany) was prepared with warm (approximately 60°C) distilled water, autoclaved (for 15 min at 120°C), cooled to room temperature, and mixed in a magnetic stirrer until the sodium alginate was completely dissolved. An aliquot of an exponentially growing algal culture was centrifuged (10 min at 2040g), washed, and resuspended in MBL medium. A selected volume of this concentrated algal cell suspension ($\leqslant 1$ mL) was then thoroughly mixed, by gentle stirring, with the alginate solution to obtain an alginate-cell suspension with the desired cell concentration. Beads were formed by extruding (dropwise) the alginate-cell suspension through a 20-mL syringe (equipped with a needle) into a 2% (w/v) aqueous solution of $CaCl_2$, from a height of approximately 15 cm and at a rate of approximately 1 drop per second. The beads were stirred in the $CaCl_2$ solution for a minimum of 45 min for gel hardening to take place. They were then washed with distilled water, stored in roughly 20-times diluted MBL medium, in the dark at 4°C, and used within 15 days of preparation. Beads had a mean diameter of 2.7 mm ($n = 79$) with a coefficient of variation of 6%. The nominal initial bead cell concentration chosen for this study was 10^6 cells/mL of alginate, because this value had been demonstrated to be suitable for the growth of immobilized *P. subcapitata* (Van Donk et al., 1992; Faafeng et al., 1994) and other species of microalgae (Hertzberg and Jensen, 1989;

Moreira dos Santos et al., 2002). The actual initial bead cell concentration estimated at the start of the in situ assay was (mean±standard deviation; $n = 3$ with 3 beads/replicate) $3.8 ± 0.46 × 10^6$ cells/mL of alginate. The immobilized cells were released within 1 h by dissolving the beads in 1 mL of a 3% (w/v) solution of trisodium citrate with the help of a vortex mixer Unimag Zx (UniEquip, München, Germany). Cell counts were conducted on three well-mixed aliquots of each replicate, under a microscope at 400 × magnification using a Neubauer chamber (American Optical, Bufalo, NY).

2.3. Study area

The area chosen to evaluate the microalgal in situ bioassay for freshwater environments was the aquatic system of the S. Domingos mine (Fig. 1), an ancient cupriferous pyrite mine located in SE Portugal. Although all mine activities ended more than 30 years ago, pyrite oxidation is still occurring in the abandoned mine tailings, continuously producing an effluent (acid mine drainage) with a pH below 3 and very high concentrations of heavy metals, mainly Fe, Al, Zn, Cu, Co, Ni, Pb, Cd, Cr, and As (Pereira et al., 1995, 2000; Lopes et al., 1999). The mine effluent is diluted by the Mosteirão stream and then discharged into the Chança reservoir. The mine aquatic system includes also two lagoons built to provide good quality water for the mine operations, known as reference sites since no other contaminant sources are present. Two reference and two impacted sites were selected to perform the in situ assay
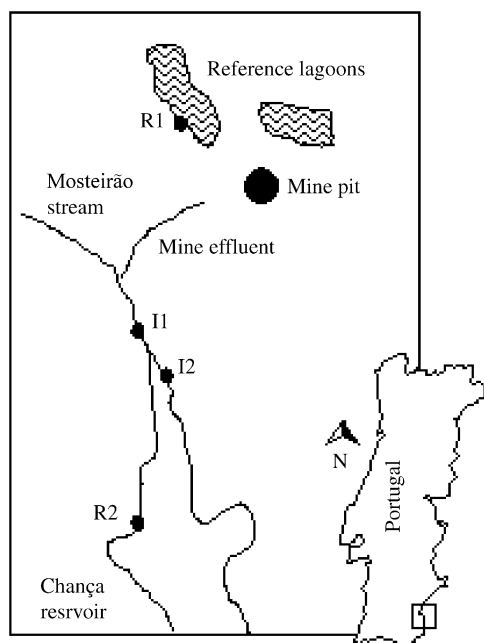

Fig. 1. Scheme illustrating S. Domingos mine aquatic system (SE Portugal) with location of reference (R1 and R2) and impacted study sites (I1 and I2).

(Fig. 1). The latter were located downstream the mine effluent discharge, one on the Mosteirão steam (I1) and the other already on the Chança reservoir (I2). The reference sites were located, one (R1) on one of the reference lagoons and another (R2) downstream of the effluent discharge, but far from the point of discharge (approx. 6 km; Pereira et al., 1995). The selection of a reference site along the same water course as the impacted sites aimed at eliminating possible differences in water quality across sites (e.g., nutrients) not associated with the drainage of the mine effluent. In this regard, it should be noted that the principal source of water flowing through the mine basin originates from the reference lagoon where site R1 was located (Pereira et al., 1995).

2.4. In situ bioassay

In developing and evaluating a microalgal in situ bioassay, one of the concerns was to follow, to the extent possible, the basic principles of standard algal toxicity tests, specifically those of the Organization for Economic Cooperation and Development algal growth inhibition test guideline (OECD, 1984). Accordingly, beads with immobilized *P. subcapitata* cells were exposed in the field for 72 h, after which time algal growth was estimated as the mean specific growth rate per day, calculated from the initial and final logarithmic (bead) cell densities, as proposed by Nyholm and Källqvist (1989). Because the specific growth rate is considered as a reproducible ecologically relevant response and not strongly test-system specific (Nyholm and Källqvist, 1989), it was selected as an appropriate test endpoint.

The bioassay apparatus designed to perform in situ assays with microalgae has been described in detailed when first developed for estuarine environments (Moreira dos Santos et al., 2002). This system, which is inexpensive to construct, has been designed to maximize simplicity and rapidity of deployment, light penetration, and water flow while preventing the entrance of organisms and mesh fouling. In short, it was composed of a plate for bead exposure (PBE) and an outer chamber to avoid damage and reduce mesh fouling and particle flocculation within the PBE. Each PBE was constructed from a 24-well microplate (Coastar, Cambridge, MA) and a sponge, so that the alginate beads were exposed tightly enclosed in wells with the top and bottom replaced by a 50-μm nylon mesh. The outer chambers consisted of rectangular shaped 5-L transparent polyethylene terephthalate bottles, cut transversally into two sections, with windows opened on each of the four sides of the top part and covered with a 200-μm nylon mesh. Control chambers consisted of closed bottles filled with control medium. Mesh and sponges were fixed with nontoxic

(Pereira et al., 1999) white-thermal glue (Elis-Tawain, TN122/WS, Tawain). Newly constructed PBEs and outer chambers were soaked in dechlorinated tap water for 24 h before being used.

Prior to deployment, measurements of temperature and conductivity (Wissenschaftlich Technische Werk-stätten LF 92 conductivity meter, WTW, Weilheim, Germany), pH (WTW 537 pH meter), and dissolved oxygen (WTW OXI 92 oxygen meter) were taken at each site. Subsurface water samples (50-cm deep) were also collected at all sites for performing the laboratory assay. The waters were filtered (50 μm) while being collected into 1-L polyethylene bottles and kept at 4°C until use. Because the goal of the current study was to evaluate had as a goal of evaluating the suitability of a microalgal in situ assay to detect an environmental impact caused by acid mine drainage rather than to identify which specific metals would have a toxic effect on *P. subcapitata*, performing metal analysis on waters from each site was not considered. Since as discussed above, acid mine drainage is known to be the sole contamination source of the aquatic system of S. Domingos mine, pH and conductivity were here used as indirect measures of the contamination intensity.

As in the previous study by Moreira dos Santos et al. (2002), logistic limitations determined that a single assay chamber and respective PBE were deployed at each study site (R1, R2, I1, I2), with subreplicates within each site consisting of eight wells. As advocated by Ingersoll et al. (1995) and observed by Moreira dos Santos et al. (2002), the lack of replication at a study site is a valid approach for identifying contaminated sites, which may then be further investigated. A control chamber was only deployed at site R2 because temperature and light conditions were similar across sites. The same medium used for stock culturing (MBL) was used as control medium.

All deployment procedures followed those previously described in Moreira dos Santos et al. (2002). In brief, beads were transported to the field in a 50-mL polypropylene beaker filled with the same medium used for bead storage. At the field site, nine beads were first preserved in Lugol's solution (Sigma Chemical, L-6146, Steinheim, Germany) to estimate the actual initial bead cell density (see Section 2.2). Then, two beads were transferred to each of eight wells and the PBE was tightly closed and placed inside the outer chamber filled with either site water or control medium. The two parts of the outer chamber were then sealed with transparent tape, the control chamber was filled with the respective medium, and the meshed chamber was filled with site water by gentle submersion. The chambers were closed ensuring that the PBE was positioned at the height of the mesh windows, by fixing a nylon wire tied to the PBE with the help of the lid. Finally, the chambers were positioned neck downward and anchored a few meters

from the shore. At the end of the 72-h exposure, each group of two beads was collected, preserved in Lugol's solution and transported to the laboratory to estimate subreplicate bead cell densities as previously described.

Upon arrival from the field, three replicate cultures of immobilized *P. subcapitata* cells were set up in the laboratory under controlled conditions, to evaluate the biological viability of the immobilized *P. subcapitata* cells and their potential to be used for in situ testing. Each replicate consisted of three beads cultured in 175-mL flasks, filled with 100 mL of MBL medium, and covered with laboratory Parafilm (American National Can, Menasha, WI) perforated with a needle to reduce evaporation but allow gas exchange. The three cultures were incubated for 72 h in an orbital shaker (LH Fermentation Series F200, Kempsters, Basingstoke, UK) at 100 rpm and 23 ± 1°C, under continuous cool-white fluorescent light (zenital disposition; 7000 lx). Final bead cell densities of each replicate culture were determined as described above.

2.5. Laboratory bioassay

A laboratory bioassay on all site waters was carried out in parallel with the in situ assay to complement the information gained from the latter, and in this way more comprehensively assess the impact of the mine effluent on microalgae. As for the in situ assay, the laboratory assay procedures closely followed the OECD guideline for algal toxicity testing (OECD, 1984). Free cells of *P. subcapitata* were used as test organism, instead of cells immobilized in beads, as required for the in situ assay. The MBL medium was likewise used as control. In addition, the assay was carried out in sterile 24-well microplates (with 1 mL of medium/well) analogous to those used to construct the PBEs. Microplate algal assays have major advantages over conventional assays: practicability, simplicity, rapidity, the need for less laboratory resources, and low volumes of samples. Their effectiveness for toxicity assessments, particularly in terms of sensitivity and reproducibility, has been demonstrated by several authors (Thellen et al., 1989; Blaise et al., 1998, and references therein; Geis et al., 2000). As a result, a guideline for a growth inhibition test with *P. subcapitata* using the microplate technique was proposed in 1992 by Environment Canada. The laboratory assay was performed using simultaneously plain site waters and site waters enriched with the same nutrients comprising the MBL. Because growth differences when performing a microalgal assay on natural water samples may be due to toxicity, nutrient shortage, or both, it is a recommended approach for laboratory algal assays evaluating the hazard of natural samples (e.g., effluents) to add nutrients to the test waters (Environment Canada, 1992; USEPA, 1994; Eklund et al., 2002). In this way, nutrient differences among

waters are eliminated and it is possible to distinguish a toxic effect from that caused by poor nutrient levels.

Like the in situ assay, the laboratory assay consisted of a 72-h growth test. All site waters were vacuum-filtered (0.45 μm) before testing to remove indigenous microalgae. Nutrient-enriched waters from each site (500 mL) were then prepared by adding all nutrients contained in the MBL medium at the same recommended concentrations. The algal inoculum, obtained in the same way as for bead preparation, was diluted to achieve a cell density of 10^5 cells/mL. Such a dilution was prepared for each treatment: control, plain, and nutrient-enriched site waters. Three replicate cultures were set up randomly for each treatment. For this, each well in the microplates was filled with 900 μL of test water and inoculated with 100 μL of the correspondent algal-inoculum solution, so that the nominal initial cell concentration was 10^4 cells/mL. Peripheral wells were excluded from the assay because evaporation in these wells is greater. Before placing the lids the microplates were covered with Parafilm to reduce evaporation. They were incubated in an orbital shaker (LH Fermentation Series F200) at 100 rpm and $23 \pm 1°C$, under continuous cool-white fluorescent light (zenital disposition; 7000 lx). As recommended by Thellen et al. (1989), the contents of each well were thoroughly mixed on each day of the test, by repetitive pipetting, to promote active gas exchange and prevent clumping of the cells. To estimate cell densities at test termination, a 500-μL sample of each replicate culture was preserved with 500 μL of Lugol's solution. Cell counts to estimate the specific growth rate were made on three well-mixed aliquots of each replicate as previously stated.

2.6. Data analysis

To investigate the biological viability of immobilized *P. subcapitata* and its potential for in situ testing, the mean specific growth rate of entrapped cells cultured under controlled conditions was compared with that of the control free cell cultures in the laboratory assay by an independent samples *t* test. Specific growth rates from the in situ and laboratory assays were compared by independent samples *t* tests assuming equal variances

(Zar, 1996). While the comparison between control-specific growth rates was made on the absolute values, for the local waters growth rates were expressed as a percentage of the control to eliminate, to the extent possible, differences in assay-system and environmental factors. Relative growth rates were arcsine-square-root-transformed prior to analysis to obtain homogeneous variances (Zar, 1996). For the laboratory assay, differences in growth rate among site waters were assessed through one-way analysis of variance (ANOVA) followed by a Tukey honestly significant difference (HSD) multiple-comparison test, within each nutritive status combination. Independent samples *t* tests were used to compare laboratory growth rates between plain and nutrient-enriched local waters within each site. Statistical significance was established at $P \leqslant 0.05$.

3. Results

The water-quality parameters measured at the time the in situ and laboratory assays were performed are given in Table 1. Temperatures were slightly higher during the in situ than during the laboratory assay: they varied from 24°C to 25°C during the in situ assay, at the reference (R1 and R2) and impacted sites (I1 and I2), respectively, while $23 \pm 1°C$ was the temperature established for the laboratory assay. During both assays, a gradient of pH and conductivity was evident from the reference to the impacted sites, and within the latter. The pH was higher and conductivity was lower at the reference than at the impacted sites. At site I1, the closest to the point of discharge, the water was acid with an almost three times higher conductivity than at site I2, where pH was nearly neutral. Dissolved oxygen measurements were always above 8 mg/L for both the in situ and laboratory assays.

The daily mean ($\pm$ standard deviation) specific growth rate of immobilized *P. subcapitata* laboratory cultures (1.05 ± 0.06, with a coefficient of variation of 5%) set up to investigate their biological viability and suitability for in situ testing was significantly lower (by 27%) than that of the free cell cultures of the laboratory assay control (1.44 ± 0.04, with a coefficient of variation of 2%)

Table 1
Water-quality parameters for each study site measured at the beginning and end of the in situ bioassay and at the beginning of the laboratory bioassay

Parameter[a]	In situ bioassay				Laboratory bioassay			
	R1	R2	I1	I2	R1	R2	I1	I2
Temperature (°C)	24	24	25	25	23±1	23±1	23±1	23±1
pH	7.45–8.11	7.20–7.19	4.00–3.40	7.34–6.41	7.32	7.16	3.02	6.67
Condition (μS/cm)	—[b]	—[b]	—[b]	—[b]	176	216	652	226

[a] Dissolved oxygen was always above 8 mg/L.
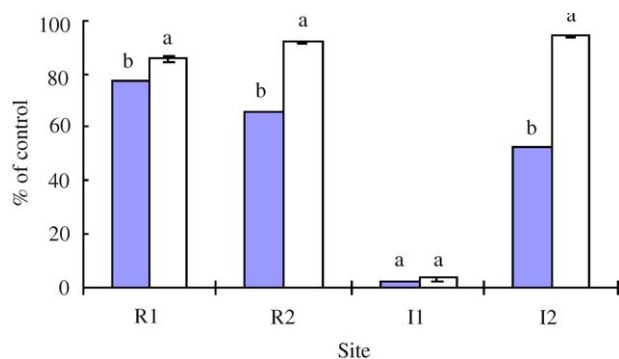[b] No values available.

Fig. 2. Specific growth rates of *Pseudokirchneriella subcapitata* expressed as a percentage of the control growth rate, for the in situ assay using immobilized cells (gray columns, $n = 1$) and laboratory assay treatments with plain site waters using free cells (white columns, $n = 3$). Error bars indicate ± 1 standard deviation; common letters above error bars indicate values not significantly different by independent samples t test assuming equal variances, when tested within each site water.
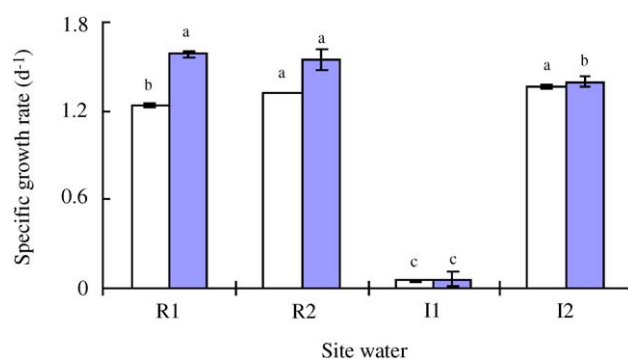

Fig. 3. Mean ($n = 3$) specific growth rates of free cells of *Pseudokirchneriella subcapitata* for the laboratory assay with plain (white columns) and nutrient-enriched site waters (gray columns). Error bars indicate ± 1 standard deviation; common letters above error bars indicate values not significantly different by Tukey HSD tests, when tested within each nutrient status combination.

($t_2 = 10.1$, $P < 0.001$). In these treatments, the cell density during testing increased by 24 and 76 times, respectively. A significant difference was also found between the in situ (1.17, with a coefficient of variation within subreplicates of 3%) and laboratory assay control-specific growth rates ($t_2 = 6.67$, $P < 0.05$). During the in situ assay, the bead cell density increased by a 33-fold factor (with a coefficient of variation of 9%).

Specific growth rates are presented in Fig. 2 as a percentage of control-specific growth rates, for the in situ and laboratory assay treatments with plain site waters. The highest in situ growth rates were observed at the reference sites; they were higher than at impacted sites by 21–98%. Within the impacted sites, immobilized *P. subcapitata* grew much more at site I2 than at I1 (by 96%). Laboratory growth was significantly different across sites ($F_{(3,8)} = 2462.2$, $P < 10^{-11}$): it was significantly higher for sites R2 and I2 than for sites R1 and I1, with the latter two values significantly different from each other. It was also observed that growth was significantly lower in situ than in the laboratory for sites R1 ($t_2 = 4.95$, $P < 0.05$), R2 ($t_2 = 364.5$, $P < 0.001$), and I2 ($t_2 = 24.9$, $P < 0.01$). For these three sites, growth in the laboratory was more than 85% of control growth, while in situ growth was higher than 65% of control growth at both reference sites and decreased to 52% of control at site I2. Conversely, growth at site I1 was equal to or lower than 3.2% of control growth whether the assay was carried out in situ or in the laboratory.

Fig. 3 presents the specific growth rates of *P. subcapitata* for the laboratory assay performed with plain site waters and with site waters enriched with the same nutrient concentrations as the MBL medium. As with the relative growth rate results, significant differences were found among plain site waters ($F_{(3,8)} = 7149.7$, $P < 10^{-13}$): growth at sites R2 and I2

was slightly higher than at site R1 (only by $\leqslant 9\%$) and largely greater than at I1 (by $\geqslant 96\%$). The addition of nutrients to the site waters significantly increased growth at the reference sites ($t_4 \geqslant 5.48$, $P < 0.05$), but not at the impacted sites ($t_4 \leqslant 1.81$, $P > 0.05$). Therefore, the significant growth differences observed among enriched site waters ($F_{(3,8)} = 754.9$, $P < 10^{-9}$) did not follow the same pattern as those among plain site waters: growth at reference sites was significantly higher than at impacted sites (by 10–96%).

4. Discussion

The specific growth rates displayed by immobilized and free cell cultures under laboratory-controlled conditions demonstrated the potential of *P. subcapitata* cells encapsulated in calcium alginate beads for conducting algal in situ bioassays in freshwater environments. Although growth was significantly higher for free than for immobilized cell cultures, immobilized *P. subcapitata* was able to grow in accordance with acceptability criteria required in various standard guidelines for control growth, namely that: (a) at the end of the exposure period (3–4 days) cell density should increase by at least a 16-fold factor (OECD, 1984; Environment Canada, 1992) for chronic effects to be expressed (Nyholm and Källqvist, 1989), and that (b) the coefficient of variation of the mean specific growth rate should be $\leqslant 20\%$ (Environment Canada, 1992; USEPA, 1994).

The present results are in agreement with those of other studies performed with either freshwater (including *P. subcapitata*) or marine microalgae, reporting better growth performance of free algae compared to their growth when immobilized in alginate beads, even though the biological viability of at least the

great majority of the entrapped cells was maintained (Bozeman et al., 1989; Santos-Rosa et al., 1989; Van Donk et al., 1992; Pane et al., 1998). This difference has been ascribed to the occurrence of growth-limiting conditions within the alginate matrix caused by restrictions in the diffusion of nutrients, carbon dioxide, and light (Robinson et al., 1985; Santos-Rosa et al., 1989; Pane et al., 1998). An additional reason for the growth differences observed here between free and immobilized *P. subcapitata* cultures was possibly the lower initial cell density established for the free than for the immobilized cultures, approximately 10^4 cells/mL of medium compared to 10^6 cells/mL of alginate. Microalgal growth rates have indeed been shown to decrease with increasing initial cell densities for both free (Nyholm and Källqvist, 1989; Franklin et al., 2002) and immobilized cell cultures (Hertzberg and Jensen, 1989; Santos-Rosa et al., 1989), due to limitations in nutrient, carbon dioxide, and light available for optimal growth. Although the cell densities studied here may not be directly comparable, it can be assumed that the cell density within the alginate matrix influences the adjacent medium as if the latter would have an equal cell density. Despite possible differences in the absolute growth rates of free and immobilized microalgae, it has been shown in the laboratory that, in general, immobilized and free cells respond similarly to contaminants (Bozeman et al., 1989; Van Donk et al., 1992; Abdel-Hamid, 1996). Such evidence strongly suggests that using *P. subcapitata* immobilized in alginate beads is a valid option for in situ toxicity evaluations.

Similar to the previous study in an estuarine environment (Moreira dos Santos et al., 2002), the assay chambers with their simple and cost-effective design allowed the easy and successful deployment of the microalga at the water surface (optimizing light availability), and its retrieval at the end of the 3-days exposure in the aquatic system of S. Domingos mine. Moreover, the fact that the increase in cell density displayed by the in situ control chamber fulfilled the growth performance criteria stated above further confirmed the appropriateness of this novel assay for routine water-quality assessments in diverse aquatic environments, and in particular the suitability of immobilized *P. subcapitata* cells for in situ toxicity testing.

According to the results of the in situ assay, *P. subcapitata* appeared to have been negatively affected by the mine effluent. Compared to the growth observed at both reference sites, there was a decrease in growth at site I2 (by 21–33% relatively to sites R2 and R1, respectively) and a pronounced decrease at site I1 (by 97–98%). The toxic hazard of acid mine drainage is mainly associated with acidity itself, high concentrations of heavy metals, and turbidity caused by suspended solids (Kelly, 1988). At both impacted sites, but particularly at site I1, some turbidity was noted during assay deployment. The values of pH measured during the in situ assay and those of conductivity measured upon arrival at the laboratory also indicate that site I1 was clearly severely impacted by the mine drainage, whereas site I2 was probably moderately impacted. The free metal ion is known as the most bioavailable and thus toxic form of a metal, and the proportion in an environment depends mainly on water-quality parameters like pH, hardness, and inorganic and organic ligands (Kelly, 1988; Gerhardt, 1993). Since the pH at site I2 was close to neutrality, it is possible that a lower acid mine drainage impact was due to the metals present (though at low concentrations, as suggested by the low conductivity) and to some turbidity. The adsorption of metals to biological surfaces is expected to increase as pH increases up to around neutrality, enhancing the chances of metal uptake by the organisms (Xue et al., 1988; Gerhardt, 1993). In addition, the complex metal interactions which generally take place within metal mixtures can produce either synergistic or antagonistic toxic effects (Gerhardt, 1993; Rachlin and Grosso, 1993), complicating the interpretation of results.

Despite the potential acid mine drainage impact suggested from the in situ responses, it is often very difficult in in situ assays to establish toxicity as the sole cause for the observed effects because of confounding factors (Chappie and Burton, 1997; DeWitt et al., 1999; Sibley et al., 1999; Ringwood and Keppler, 2002; Castro et al., 2003). Such factors are mainly associated with the transport and caging of the test organism, spatial and temporal variations in the toxicant concentration, and environmental conditions (e.g., light, temperature, and nutrient concentrations). As the in situ control growth of *P. subcapitata* was within acceptability criteria, it is not very probable that the transportation of the beads to the field had any detrimental effect on the health status of the alga. With regard to caging, the fact that some mesh fouling and accumulation of fines was observed at the end of the 3-day exposure, particularly at the two impacted sites (I1 and I2), should be taken into consideration. Such occurrences may have reduced the penetration of light through the chambers at these sites, though it is not likely that they are the sole reason for the growth inhibition seen at least at site I1. Increasing the mesh size for future studies may pose problems of bead destruction due to the entrance of indigenous organisms. Thus, a feasible way to avoid problems due to fouling would require the frequent brushing of the meshes, if possible once a day. Because the stability of alginate gels is known to be largely affected by cation chelators (e.g., phosphate, citrate) (Dainty et al., 1986; Martinsen et al., 1989), bead disruption with the resultant loss of algal cells to the medium could constitute another confounding factor. However, this phenomenon is potentially more relevant

in marine/estuarine waters which are particularly rich in dissolved and particulate organic matter (Moreira dos Santos et al., 2002). Also, bead disruption is not expected to take place under such short test durations (3 days) (Faafeng et al., 1994; Moreira-Santos, personal observation). Since the values of pH registered at the start and end of the in situ assay were relatively stable within each of the four sites, it is suggested that there were no substantial variations in the exposure regime during testing, and consequently that the drainage of the mine effluent reached site I1 and most probably also site I2. The only environmental condition that could not be controlled across sites was the nutrient status, since it is not possible to identify/measure all the nutritional constituents of local waters. Nevertheless, according to the location of the study sites, nutrient levels across sites R2, I1, and I2 should be largely the same and no substantial differences were expected between site R1 and all the remaining sites. Minimal (1°C) water temperature differences were found across sites whereas the four study sites had been selected so that all assay chambers were deployed under the same light conditions (no field site had canopy cover).

Somewhat unexpectedly, in the laboratory assay designed to more comprehensively interpret the results of the in situ assay the response pattern of *P. subcapitata* cultured in plain site waters did not agree with that of the in situ assay. Growth rates in waters from sites I2 and R2 were similar, slightly higher than in R1 water and much greater than in water from I1. Such an outcome was a consequence of the fact that the significant increase in algal growth in the laboratory relatively to the field was not of the same magnitude for all sites. Even though the comparison between in situ and laboratory results was based on growth rates normalized to percentages of the control values, apparently this procedure was not sufficient to eliminate all procedural and environmental differences between the assays. It is well known that the application of strategies to distinguish the effects of environmental variables from those of contaminants should not rule out possible interactions between these factors (DeWitt et al., 1999; Maltby et al., 2002; Moreira dos Santos et al., 2002; Ringwood and Keppler, 2002). The differences observed in *P. subcapitata* growth between in situ and laboratory controls had been anticipated due to the dissimilarities in the light regime conditions; light/dark cycle versus continuous light, respectively. However, the more favorable light conditions during the laboratory assay may not have been equally expressed for all site waters. It is possible that by reducing the restrictive effect of turbidity for algal growth due to the use of small volumes of water and because site waters were filtered prior to use, the laboratory assay enabled a more pronounced increase in growth at site I2 than at the reference sites. Because no definitive conclusions on

the influence of the light regime on microalgae sensitivity to toxicants have been reported (Nyholm and Källqvist, 1989), it is not possible to speculate upon the possible effects of this factor on the *P. subcapitata* responses observed in the present study. A decrease in the bioavailability of the metals leading to higher growth rates in site I2 water may have also occurred in the laboratory assay due to two possible reasons: (1) the high cell densities resulting from the elevated growth rates may complex available metals leading to a lower metal load and thus less metal uptake per individual cell and its exudates (Wang and Dei, 2001; Franklin et al., 2002); and (2) by using small test vessels (like microplate wells) the large contact area per volume ratio may substantially contribute to the adsorption of metals to the walls of the well decreasing the metals available for cellular uptake (Hörnström, 1990). Neither of these two situations is likely to occur under field conditions since the complete depletion of metals from the surrounding water by surface adsorption would be nearly impossible. Eventually, confounding factors such as those discussed earlier may also be at the origin of differences between responses from in situ and laboratory assays. In particular, the limitation of the water samples collected at the field sites for performing the laboratory assay to actually represent the natural toxicant exposure regime due to spatial and temporal variations during testing is often times responsible for discrepancies found between in situ and laboratory results (Moreira dos Santos et al., 2002, and references therein). However, such circumstances are not expected to have happened since as previously discussed the aquatic system of S. Domingos mine was considered to be reasonably stable during the 3-day in situ assay period.

Performing the laboratory assay using simultaneously nutrient-enriched and plain site waters revealed that the addition of nutrients ameliorated *P. subcapitata* growth at the reference sites but not at the impacted sites. Because there is no reason to believe that there were major nutrient discrepancies across sites, the lack of an increase in growth at sites I1 and I2 suggests that nutrients were at least not the sole factor limiting the growth of *P. subcapitata* in the field. Thus, this finding corroborates the results of the in situ assay in which sites I1 and I2 appeared, respectively, as severely and moderately impacted by the acid mine drainage. Furthermore, these results evidence the need to ensure a similar composition of essential nutrients across treatments in order to better interpret results of microalgal assays evaluating the hazard of natural water samples. However, it should be taken into consideration that the nutrient status influences the sensitivity of algae to pollutants. Exposure at high nutrient concentrations has been shown to increase or decrease the sensitivity, as well as to have no influence on toxicity, depending on the species, strain, the metal, and its mode of action

(Rijstenbil et al., 1998; Nalewajko and Olaveson, 1998; Wang and Dei, 2001). The elucidation of these mechanisms whenever required should be included as an important stage in each particular integrative assessment study.

5. Conclusions

This study demonstrated that the in situ bioassay chambers and procedures previously developed for estuarine environments (Moreira dos Santos et al., 2002) were suitably adapted to evaluate toxicity in freshwater lentic systems. The microalga *P. subcapitata* immobilized in calcium alginate beads grew well and according to acceptability criteria when deployed in a control chamber. Moreover, the in situ assay design was able to identify the sites impacted by the drainage of the mine effluent, confirming assay sensitivity and its potential as an effective tool to be incorporated in ecological risk assessment studies. By discriminating effects associated with site differences in nutrient levels from those due to the impact of the mine effluent, the laboratory assay conducted following standard guidelines permitted substantiation of the in situ results. Along with other investigators (Maltby and Crane, 1994; Culp et al., 2000), this study demonstrated that an integrative hazard assessment approach that combines information from both in situ and laboratory assays, as well as water-quality parameters, will increase the ecological realism of the resultant environmental decisions. Such an approach is particularly relevant for moderately impacted environments where the establishment of a causal link between exposure and effects is more complex (Clements and Kiffney, 1994; Pereira et al., 1999; Cherry et al., 2001).

Acknowledgments

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