A PHYTOPLANKTON GROWTH ASSAY FOR ROUTINE IN SITU ENVIRONMENTAL ASSESSMENTS

MATILDE MOREIRA-SANTOS,† AMADEU M.V.M. SOARES,‡ and RUI RIBEIRO*†
†Instituto do Ambiente e Vida, Departamento de Zoologia da Universidade de Coimbra, Largo Marquês de Pombal, 3004-517 Coimbra, Portugal
‡Departamento de Biologia da Universidade de Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
(Received 11 July 2003; Accepted 18 November 2003)

Abstract—This study proposes an ecologically relevant and cost-effective phytoplankton growth assay for routine in situ toxicity assessments. Assay procedures were developed applying, to the extent possible, the rationale behind the design of standard algal assays. Chlorella vulgaris was selected as test species because it grows well immobilized in alginate beads and has a wide geographic distribution. The performance of the assay in a freshwater system impacted by acid mine drainage demonstrated the suitability of assay chambers and procedures. The test system, made of inexpensive materials, allowed the rapid and easy deployment of the assay. The deployment of extra chambers at reference sites provided the ability to periodically check whether algal growth had already reached recommended growth criteria (time at which the assay should end). By deploying chambers filled with control medium at all sites, temperature was identified to explain 95% of the variation in growth. By using an artificial nutrient source shown capable of promoting algal growth according to recommended standards, toxicity from the mine effluent was distinguish from in situ nutrient limitation effects. The very good agreement ($r^2 = 0.90$) between mean in situ growth rates estimated by microscopy and by spectrophotometry and their similar coefficient of variation showed the latter to be a suitable straightforward methodology for assay endpoint estimation.

Keywords—In situ bioassay Immobilized microalgae Growth inhibition Chlorella vulgaris Test development

INTRODUCTION

The search for ecological relevance in risk assessment studies in past years has led to the development of a new generation of bioassays to determine pollutant effects in situ. Because test organisms are exposed at field sites, in situ assays integrate much of the natural fluctuating environmental conditions, being ecologically more relevant than laboratory assays and offering greater experimental control than field studies [1–5]. In situ assays can be particularly relevant as part of integrative toxicity evaluations to establish causation in ecological risk assessment [6–8].

Despite being recognized as valuable tools for assessing the environmental hazard of substances, in situ assays have not yet been widely implemented, either in ecological risk assessments or in monitoring programs, mainly due to their complexity and high costs. In addition, these are the probable reasons for the low number of studies concerning the use of phytoplankton for in situ toxicity evaluations, when compared to in situ assessments using aquatic animals [3]. Because phytoplankton is an ecologically important group in most lentic as well as lotic aquatic systems, known to be relatively sensitive to pollutants [9–11], guidelines for toxicity testing with microalgae have been established [12–14] and are endorsed formally for regulatory purposes [10,15]. These standard laboratory algal assays have been designed so that, by providing an optimum set of experimental conditions, mainly in terms of light, temperature, carbon dioxide and nutrients, exponential growth can be maintained in control cultures for 72 h (see Nyholm and Källqvist [9] for a detailed discussion on algal test methods).

Applying the rationale associated with the design of standard algal assays to algal in situ assays is complex and needs to be adjusted. The immobilization of algal cells in a matrix of calcium-alginate gel recently has been shown to be a suitable methodology for exposing the planktonic algae to the continuously changing field conditions without the risk of being washed out or lost by predation, sedimentation, or biofouling [3,16,17]. Yet, because algal growth is very much dependent on environmental factors [9,18], two other major issues have to be considered in the design of an in situ algal assay. First, the occurrence of low temperatures in the field, particularly in temperate regions, and the nutrient limiting conditions that exist in most aquatic systems are examples of important factors restricting algal growth at reference sites. These adverse conditions may compromise the achievement of the criterion recommended in standard protocols and consequently the detection of toxic effects in general [9]. Second, because environmental factors at reference and contaminated sites are seldom similar, it is generally very difficult to ascribe the in situ response exclusively to the effects of the contaminant(s). Approaches for discriminating environmental effects from contaminant effects are required and so far have been considered in only a few in situ assays [1,3,4,19].

AWARE of the need to incorporate phytoplankton in situ toxicity evaluations in water quality assessments and biomonitoring, this study had two main objectives. The first was to develop procedures for an ecologically relevant and cost-effective in situ phytoplankton growth assay, suitable for routine toxicity assessments. The second objective was to evaluate the potential of the developed assay for in situ toxicity assessments.
in both lotic systems (in terms of general performance) and lentic systems (by carrying out an assay at an acid mine drainage impacted area). To achieve the first objective, a series of laboratory experiments were conducted according to four specific tasks. The first was to select a species of green algae yielding optimal growth when immobilized in beads of calcium alginate. Second was to investigate the use of a source of nutrients during exposure as a mean to discriminate toxic effects from those due to nutrient limitation, and simultaneously to promote algal growth at reference sites in accordance to recommended standards. Third was to examine the influence of factors determinant for algal growth (initial bead cell density and temperature) in an attempt to infer upon the duration of the assay required for recommended growth standards to be achieved. Fourth was to explore the use of spectrophotometry as a straightforward method to estimate the assay endpoint.

MATERIALS AND METHODS

Test organisms

The two species of green microalgae used in this study were Pseudokirchneriella subcapitata (Korshikov) Hindak (previously named Raphidocelis subcapitata Korshikov and Selenastrum capricornutum Printz) and Chlorella vulgaris Beijerinck. Both were obtained from the Carolina Biological Supply (Burlington, NC, USA), and maintained in nonaxenic batch cultures in 250-ml erlenmeyer flasks containing 100 ml of Woods Hole Marine Biological Laboratory (Woods Hole, MA, USA) growth medium [20] (hereafter referred to as MBL medium), at 20 ± 1°C under continuous cool-white fluorescent illumination (100 μE/m²/s). To start new cultures, algae were harvested while still in the exponential growth phase (5–7 d old). Both species were maintained under these culture conditions for at least six months before conducting experiments.

Cell immobilization

The procedure for immobilization of algal cells in beads of calcium alginate was adapted from Moreira dos Santos et al. [3]. A 1.3% (weight/volume) sterile solution (autoclaved 15 min at 120°C) of sodium alginate (A-7128; Sigma Chemical, Steinheim, Germany) was mixed with an inoculum of algal cells (≤1 ml) to prepare an algal-cell suspension with the required cell concentration. To obtain the algal inoculum, an aliquot of an exponentially growing culture was harvested, centrifuged (10 min at 3,500 rpm) and resuspended in MBL medium. The beads were formed by forcing this mixture through a syringe (fitted with a needle) into a 2% (w/v) aqueous solution of CaCl₂. After being washed with distilled water, beads were stored in diluted MBL medium (~20 times) in the dark at 4°C for no more than 15 d. Beads prepared in this way had a mean diameter between 3.0 and 3.6 mm (n = 50–100) with coefficient of variation (CVs) of 4 to 5%.

Laboratory growth experiments

The laboratory experiments followed the Organization for Economic Cooperation and Development (OECD) guidelines for algal growth inhibition tests [12], with modifications with respect to algal inoculum, culture medium, and test recipients. The nominal initial bead cell concentration was approximately 10⁶ cells/ml of alginate, except when stated otherwise. This cell concentration has been demonstrated as suitable for the growth of immobilized microalgae [3,17,21]. Two standard media were used as control, the MBL [20] and the one recommended by the OECD [12]. Although the MBL is a richer medium than the OECD, both media present a similar nitrogen/phosphorous ratio (~10). Unless otherwise stated, experiments were conducted in 175-ml glass vials filled with 120 ml of medium, with cultures randomly incubated in an orbital shaker (LH Fermentation, Series F200, Kempters, Basingstoke, UK) at 100 rpm. The vials were covered with laboratory Parafilm (American National Can, Menasha, WI, USA) perforated with a needle (~6 times) to reduce evaporation but allow gas exchange. All experiments consisted of 72-h growth tests, except when experimental duration was an independent variable. The endpoint chosen to estimate algal growth was the specific growth rate per day, calculated from the initial and final (bead) cell densities [9]. To estimate bead cell densities, beads were preserved with Lugol’s solution (L-6146; Sigma Chemical) at the beginning and end of each experiment. Immobilized cells were released (within 1–2 h) by dissolving the beads in a 6% (w/v) solution of trisodium citrate (≤1.5 ml), and counted under a microscope at ×400 magnification using a Neubauer chamber (American Optical, Buffalo, NY, USA).

Selection of test species

This first experiment aimed at selecting a species of planktonic green algae with a good growth performance when immobilized in alginate beads and, at the same time, appropriate for in situ toxicity evaluations on a large geographical scale. Pseudokirchneriella subcapitata and C. vulgaris were chosen because they are both readily available and easily cultured under laboratory conditions, and widely used and recommended for (standard) toxicity testing [12–14]. Specific growth rates of immobilized cells were evaluated using MBL as control medium. Reference water from site R1 (see Study Area) (pH = 7.64; conductivity = 197 μS/cm) without and with nutrient additions (R and R+N, respectively) also was used as growth medium, to simulate a range of field growth responses. Upon collection, the R water was vacuum filtered (0.45 μm) to remove indigenous algae, and kept in darkness at 4°C until use. The R+N medium was prepared by adding all nutrients contained in MBL medium at the same recommended concentrations. For each algal species–medium combination, three replicates with three beads with initial cell densities of 1.6 × 10⁶ (P. subcapitata) and 1.3 × 10⁶ cells/ml of alginate (C. vulgaris) were set up. Cultures were incubated at 23 ± 1°C under continuous cool-white fluorescent light (100 μE/m²/s). Results obtained determined the selection of C. vulgaris to be used hereafter.

Exposure with a nutrient source

The feasibility of using a source of nutrients during exposure was investigated as a way to distinguish toxic effects from those due to nutrient limitation. The use of an artificial fertilizer (Blaukorn, 0008, Aventis CropScience, Lisboa, Portugal) as a source of macro- (N, P, K, Ca, Mg, S) and micro-nutrients (B, Mn, Zn) was evaluated using the in situ test system (see Evaluation I: Assay potential for assessments in lotic systems) as test recipients, to better simulate an in situ assay. The OECD medium was selected as control because it presents nutrient concentrations assumed to more realistically represent a broad range of environments than the MBL medium. A single treatment (ASTM+F) consisting of the American Society for Testing and Materials (ASTM) hard water [22] 20 times diluted (with distilled water) plus 60 g of fertilizer per replicate was set up. The ASTM hard water was diluted to yield a medium with low salt concentrations, because
preliminary experiments showed the full strength medium to be capable of sustaining algal growth. Before the experiment, each 60 g of fertilizer particles was thoughtfully mixed with distilled water (500 ml) for 45 s with the help of a metal spatula, and washed through a 1-mm nylon screen to remove their blue coating. They were then dried in an oven (4 h at 40°C) to prevent their immediate dissolution when added to medium. Preliminary experiments showed that, when in contact with water, the blue coating from the fertilizer particles originates small fragments that either remain in suspension or sink, covering the alginate beads and decreasing the light available for algal growth. Three replicates were run for each of the two treatments (OECD and ASTM+F). For each replicate, three sets of 30 beads with immobilized C. vulgaris at an initial concentration of $0.55 \times 10^6$ cells/ml of alginate were placed on a chamber for bead exposure. The in situ test chambers were randomly placed (floating) inside plastic trays filled with tap water. They were incubated at $25 \pm 1°C$ under continuous cool-white fluorescent light (60 $\mu$E/m$^2$/s), to maximize the requirement for nutrients and fully evaluate the suitability of the fertilizer. Twice a day chambers were gently shaken by hand to help mix the fertilizer in the water column.

**Assay duration—initial bead cell density vs temperature**

The influence of initial bead cell density and temperature on the growth of immobilized C. vulgaris was examined to better understand the role of these two algal growth determinants and their interaction on the ideal duration of an assay [12,13]. The initial cell density is an important factor in algal assays performed either with free cell suspensions [9,23] or immobilized cells [21,24] because the density of cells in the medium and particularly in the alginate matrix influences the nutrient, carbon dioxide, and light available for optimal growth. Beads with a low and a high density of C. vulgaris cells were prepared, $2.0 \times 10^6$ and $2.1 \times 10^6$ cells/ml of alginate, respectively. Previous studies showed such cell densities to be suitable for the growth of immobilized microalgae [3,17,21]. For each initial cell density, beads were incubated at four different temperatures, 10, 15, 20, and 25°C. To allow the cell density in all treatments to increase by at least the recommended factor of 16-fold, the experimental duration was adjusted for each temperature, 11 (a longer duration was not possible for practical reasons), 9, 7, and 3 d, respectively. The MBL medium was used as growth medium to ensure that inhibition of growth due to nutrient limitation would not occur during testing. For each initial bead cell density—temperature combination, three replicate cultures of 30 beads each were incubated under cool-white fluorescent light (100 $\mu$E/m$^2$/s) with a regime of 12:12-h light:dark. In this experiment, specific growth rates were estimated throughout the incubation period on days 3, 5, 7, and 9, except for the 10°C trial for which estimations were made only on day 11. Three extra replicates were set up for cell counts on each assessing day. Because the four different temperatures were not run simultaneously and beads had to be prepared more than once, a control to check for the existence of similar basic experimental conditions (e.g., health of algal inoculum, quality of medium) was set up for each initial bead cell density—temperature combination. These controls consisted of three replicates of five beads incubated at $20 \pm 1°C$ under continuous cool-white fluorescent light (60 $\mu$E/m$^2$/s) during 5 d.

---

**Assay endpoint estimation—microscopy vs spectrophotometry**

This part of the study aimed at exploring the use of spectrophotometry as a simple and rapid methodology for assay endpoint estimation by comparing specific growth rate results obtained from microscopic countings and spectrophotometric absorbance readings. For this, all replicate cultures from the experiment described in the previous task were used. The only complement to the referred experimental design was related to the need to adjust the blank reading of the spectrophotometer. An additional set of 30 blank beads (beads not inoculated with C. vulgaris cells) was incubated for each treatment under exactly the same conditions. With respect to the procedures for cell density estimation prior to bead dissolution, the Lugol's solution in which each group of beads had been preserved was replaced with dechlorinated tap water (~10 ml), and the beads were kept in the latter for 1 h. This procedure was intended to wash the beads because the presence of Lugol's solution can interfere with the absorbance readings, leading to high variability in the results. Microscopic cell counts were then performed as described previously. The spectrophotometric readings were performed on a Jenway 6100 Spectrophotometer (Jenway, Felsted, UK) fitted with a 1-cm cuvette to determine the absorbance (on a well-mixed 1-ml aliquot) at a wavelength of 750 nm.

**In situ evaluations—assay chambers and general procedures**

The assay apparatus for in situ assays with immobilized microalgae was specially designed to optimize light penetration and ease of deployment. It included a chamber for bead exposure (CBE) and an outer chamber (Fig. 1). Whereas the former was designed to expose the alginate beads, the latter was designed as a cage for the CBE to protect it from possible damage and reduce problems associated with particle fouling and mesh fouling. The CBE was composed of two acrylic plates, each with four holes covered on one side by a 64-$\mu$m nylon mesh. Cavities for placing the beads were formed by joining the two plates with the mesh to the outer sides. A 64-$\mu$m mesh was selected to allow adequate water flow and minimize fouling while preventing the entrance of organisms. The outer chamber consisted of a 1.1-L white-translucent plastic box (Tupperware, A02, Abrantes, Portugal) with two side
windows and nearly all the lid made of a 200-μm nylon mesh. A structure made of plastic-coated electric wire was glued inside the outer chamber for positioning the CBE at the height of the windows. Floaters consisting of three capped plastic tubes were glued on each side of the lid to reinforce the buoyancy of the outer chamber in the appropriate position. A ring made of nylon rope was glued on one of the sides of the chamber for tying it to the margin. Control assay chambers were not provided with meshed side windows and were closed tightly by introducing a piece of thin transparent plastic under the meshed lid. A nontoxic [2] white-thermal glue (Elis-Tawain, TN122/WS, Tawain) was used to fix all materials except the thin piece of transparent plastic (fixed by the lid). New CBEs and outer chambers were soaked in tap water for 24 h before use.

As part of a comprehensive approach, the in situ assay includes the deployment of three treatments at each study site. One consists of control (closed) chambers filled with OECD medium to determine to what extent differences in abiotic conditions across sites influence C. vulgaris growth. Another comprises open chambers exposing the algae to the local surrounding water (LW), and the third treatment also consists of open chambers exposing the algae to the environment of the site, but with fertilizer particles as an additional nutrient source (LW+F), in accordance with the rationale described before. At each site, each treatment was deployed in triplicate, with each replicate consisting of one CBE and respective outer chamber. The beads were transported to the field already in the CBEs inside plastic boxes filled with the same medium used for bead storage. One set of 30 blank beads (for spectrophotometric growth estimations) and three sets of 30 inoculated beads were placed on the four meshed cavities of each CBE. The set up of three subreplicates within a replicate reduces within-assay response variability and thus enhances the power of the assay to detect toxicity [25]. At the same time, three replicates of 30 beads each plus 30 blank beads were preserved in Lugol’s solution to estimate the initial cell density. At the field sites, each CBE was placed inside the outer chamber by inserting it on the electric wire structure (Fig. 1). Control assay chambers were filled with standard medium while meshed chambers were submerged until becoming filled with 200-μm filtered site water. At this stage, the storage medium still inside the cavities of the CBEs was replaced and the assay apparatus cleaned. Nine assay chambers, corresponding to triplicates of the control, LW and LW+F treatments were deployed at each site. The gradient of current velocity along which the five sites were selected was measured at deployment. Additional floaters had to be added on to ensure chambers’ buoyancy in strong currents. The experiment was carried out for 10 d. On the fifth day, current velocity was measured and the test system was checked for chamber stability. Also, all chambers were opened numerous heavy metals [26,27]. Aside from the mine effluent, no other significant pollution sources are present in the area. South of two reference lagoons (A and B), the effluent is slightly diluted by the Mosteirão stream, which flows into the reservoir of the Chança River (Fig. 2). For the in situ assay in a lentic system, three reference and two impacted sites were selected. The impacted sites were all located downstream the mine effluent discharge already on the Chança Reservoir (S1 and S2). They were chosen based on pH and conductivity values, which on the day of deployment ranged from 4.46 to 4.74 and from 421 to 354 μS/cm, respectively. Reference site R1 was situated on lagoon A, R2 was situated upstream the effluent discharge on the Mosteirão stream, and R3 was located downstream on the Chança Reservoir, far from the point of discharge. Five reference sites along a gradient of current velocity were chosen to evaluate the potential of the in situ assay in a lotic system, one on the Chança Reservoir (R3) and the remaining four on the Chança River (R4 to R7; from lowest to highest current velocity). No field site had canopy cover and light exposure was similar on all sites.

**Evaluation I: Assay potential for assessments in lotic systems**

The potential of the developed assay for toxicity assessments in lotic systems was evaluated in terms of its general performance, namely physical resistance of the system, buoyancy of chambers and bead stability, as well as of the feasibility of the frequency with which the nutrient source should be replaced and the assay apparatus cleaned. Nine assay chambers, corresponding to triplicates of the control, LW and LW+F treatments were deployed at each site. The gradient of current velocity along which the five sites were selected was 0.00 (R3), 0.02 (R4), 0.33 (R5), 0.50 (R6), and 1.20 m/s (R7). These values were measured at deployment. Additional floaters had to be added on to ensure chambers’ buoyancy in strong currents. The experiment was carried out for 10 d. On the fifth day, current velocity was measured and the test system was checked for chamber stability. Also, all chambers were opened
to renew site water and clean all meshes. The fertilizer particles were entirely replaced by a complete new dose in case they had already dissolved by more than the half.

**Evaluation II: In situ assay in a lentic system**

The developed approach was further evaluated by performing a 21-d in situ assay with immobilized *C. vulgaris* in the aquatic system of the S. Domingos mine (at three reference and two impacted sites). The three treatments were deployed in triplicate at the five sites. Because ideally the assay should end only when at the reference sites the bead cell density has increased by a factor of at least 16 (as required for control cultures in laboratory assays [9,12,13]), three extra chambers were deployed at each reference site (using treatment LW + F) to check for the increase in bead cell density (by microscopic counting) at days 6, 12, and 18. In accordance with the results obtained in the laboratory experiments, the initial bead cell density for this part of the study was 0.55 × 10⁶ cells/ml of alginate. During the assay, all chambers were cleaned and site water renewed every 3 d (day 3, 6, 9, 12, 15, and 18) to avoid reductions in water flow and light availability. The nutrient source and OECD medium were completely renewed at days 6, 12, and 18.

At each site, pH (Wissenschaftlich Technische Werkstätten 537 pH meter, WTW, Weilheim, Germany), conductivity, and temperature (Wissenschaftlich Technische Werkstätten LF 92 conductivity meter) were measured at deployment (day 0). Measurements of pH and conductivity also were taken every three days until the end of the assay. Temperature was monitored for the entire assay duration using a minimum–maximum thermometer, but readings were made only every 3 d. At the same dates, subsurface water samples were collected into 1.5-L polyethylene–terephthalate bottles and stored at 4°C for chlorophyll-α, total suspended solids (TSS), nutrient, and heavy metal analysis. Water samples were filtered (64 μm) prior to all analysis except the chlorophyll-α and TSS. Chlorophyll-α concentrations were determined immediately upon arrival to the laboratory [28]. Total suspended solid measurements followed standard methods [29]. Ammonia-, nitrate-, and nitrite-nitrogen; reactive phosphorus; and silica concentrations were measured using a DR/2000 HACH spectrophotometer (HACH, Loveland, CO, USA). Water samples for total metal analysis were preserved by the addition of nitric acid (HNO₃) until a pH ≤ 2. Heavy metal analyses were performed by inductively coupled plasma spectrophotometry [29] on composite water samples prepared from the eight water collections made at each site. Aluminum, Cd, Co, Cu, Ni, Pb, and Zn were analyzed because they are known to be among the most concentrated in the acid mine drainage impacted aquatic system [26,27] and those most likely to reach toxic levels affecting microalgae [30–32].

**Data analysis**

A major concern in this study was to develop, to the extent possible, a short-term sublethal in situ assay with microalgae in accordance to existing standard laboratory guidelines [12–14]. Therefore, two control growth criteria currently applied in ecotoxicological studies with algae were adopted in this study when appropriate. The first criterion was that cell density should increase by a 16-fold [12,13] or an eight-fold factor, because such increases correspond to three and two generations, respectively [9], and the second that the coefficient of variation of the mean specific growth rate should be ≤20% [13,14].

Laboratory specific growth rates of *P. subcapitata* and *C. vulgaris* were compared using independent samples *t* tests (for species differences within each medium) and one-way analysis of variance (ANOVA) followed by Tukey honest significant difference multiple-comparison tests (for medium differences within each species). The influence of an artificial nutrient source on the growth of *C. vulgaris* was tested by nested ANOVA [33]. A stepwise multiple-linear regression was used to determine relationships between specific growth rate and the variables initial bead cell density and temperature. The effects of these factors on *C. vulgaris* growth were analyzed using a two-way ANOVA followed by contrast analysis to test for the effects of one factor within the other [33]. For the latter analysis, replicates within each initial bead cell density–temperature combination only included specific growth rate values estimated at the end of exposure.

To explore the use of spectrophotometry, model I linear regression analyses were used to draw calibration curves of absorbance (transformed to the square root of A plus the square root of A + 1) against cell density (cells/ml of alginate log₁₀ transformed) [33]. For the laboratory experiment, a calibration curve was fitted each time a new set of beads was prepared, because absorbance is a function of cell morphology and physiology [14]. Two calibration curves were established (for cell densities ranging from 5 × 10⁵ to 3 × 10⁶ cells/ml of alginate; reliable readings were obtained only for densities ≥5 × 10⁵ cells/ml of alginate) with r² ≥ 97%. For evaluating the in situ results, an internal calibration curve was drawn for each treatment because absorbance at a wavelength of 750 nm also is dependent on the existence of dissolved and particulate organic and inorganic materials that still can be present after bead dissolution. The r² values for each of the three calibration curves were 98 (control), 94 (LW), and 90% (LW+F). For both the laboratory and in situ data, a model II linear regression was performed to investigate the relationship between mean specific growth rates determined by microscopy and spectrophotometry and Student’s *t* statistic to test if the regression slope was significantly different from 1.000 [33]. Spearman rank correlation (*r*) was used to evaluate the association between the respective *CV* values. The two sets of *CVs* were further compared by an independent samples *t* test. Prior to the latter analysis, *CVs* were arcsine square root transformed to obtain homogeneous variances [33].

For the in situ assay in the lentic system, a stepwise multiple-linear regression was used to investigate the relationship between the multiplication factor of the bead cell density in the LW+F chambers relative to day 0 (in transformed) and growth determinant factors (minimum- and maximum-temperature, TSS, and assay duration). Independent variable data used for this analysis were means of the values registered up to each exposure duration. To evaluate the effectiveness of the use of a nutrient source, control and LW+F (21-d) growth rates of *C. vulgaris* at each reference site were compared by nested ANOVAs. The relationship between 21-d control growth and abiotic factors (minimum- and maximum-temperatures and TSS) at the different sites (*n = 5*) were investigated using stepwise multiple-linear regression analysis. Because differences in minimum-temperature across sites significantly explained the in situ variation in control growth, this temperature effect was removed to the remaining two treatments (LW and LW+F) to allow the sole assessment of the impact of the
mine effluent on the growth of C. vulgaris. Both LW and LW+F relative specific growth rates were calculated as a proportion of the growth rates predicted by the regression model for each site-specific minimum-temperature. Differences in relative growth rates among and within sites (between treatments) were evaluated by nested ANOVAs followed by Tukey honest significant difference tests.

RESULTS

Selection of test species

The P. subcapitata growth rate was significantly higher than that of C. vulgaris (by 16%) only in MBL ($t_{1,4} = 3.4, p < 0.05$) and R+N media ($t_{1,6} = 6.5, p < 0.01$). The growth of both algae was significantly influenced by the culture medium ($F_{2,6} \geq 36.6, p < 0.001$); culturing in R (~0.6 d⁻¹) caused a significant growth rate decrease compared with MBL (~1.0 d⁻¹) and R+N cultures (~1.2 d⁻¹). For both species, control and R+N cultures met the validity criteria; cell density increased by a factor ≥18 and the CV of the mean growth rate was ≤10%.

Exposure with a nutrient source

Mean C. vulgaris growth rate was significantly higher ($F_{1,4} = 23.0, p < 0.01$) in control medium (OECD: 1.2 d⁻¹) than when an artificial source of nutrients was used (ASTM+F: 0.9 d⁻¹). Nevertheless, both media met the adopted acceptability criteria; cell density increased by more than 16 times and the CVs of the mean growth rates were 4 and 12%, respectively.

Assay duration—initial bead cell density vs temperature

Mean growth rates of control cultures set up at 20°C for the low initial cell density beads varied from 0.88 to 0.92 d⁻¹ with CVs ≤ 1%, whereas mean growth rates of correspondent controls for the high initial cell density beads ranged from 0.66 to 0.77 d⁻¹ with CVs ≤ 4%. Figure 3 presents mean growth rates of C. vulgaris cultured under different initial bead cell density—temperature combinations. The stepwise multiple-linear regression showed that initial bead cell density and temperature explained 85.7% of the variation in growth rate among treatments, and that 77.3% of this variation could be explained by temperature alone. Both initial bead cell density and temperature influenced significantly the growth rate of C. vulgaris, though temperature had a more pronounced effect ($F_{1,16} = 463.8, p < 10^{-16}$) than the former factor ($F_{1,16} = 19.3, p < 10^{-4}$). A significant interaction effect was also observed ($F_{1,16} = 27.9, p < 10^{-3}$). Whereas the effect of temperature was observed irrespective of the initial cell density in the beads ($F_{1,16} \geq 237.5, p < 10^{-13}$), using beads with a low instead of a high initial cell density had a positive significant effect on C. vulgaris growth only for beads incubated at intermediate temperatures (15°C: $F_{1,16} = 4.74, p = 0.045$; 20°C: $F_{1,16} \geq 93.1, p < 10^{-5}$).

The time required for C. vulgaris to achieve a mean and 95% confidence interval increase in population density equal to or higher than an eight- to 16-fold factor was determined for each initial bead cell density—temperature combination, excluding the 10°C treatments for which such increases were not attained even after an 11-d exposure. For both initial bead cell densities, the time required for such increases in population density to be accomplished decreased (by 2–4 d) with each 5°C increase in temperature; 7, 5, and 3 d were required for an eight-fold increase at 15, 20, and 25°C, respectively, and 9, 7 to 5, and 3 d for a 16-fold increase. Within each temperature, the same experimental duration was needed for the low and high initial cell density beads to achieve an eight- and 16-fold increase in population density, except at 20°C for which a 16-fold increase was achieved 2 d earlier by the low than by the high initial bead cell density.

Assay endpoint estimation—microscopy vs spectrophotometry

The relationship between mean laboratory growth rates of C. vulgaris determined by microscopy and spectrophotometry is plotted in Figure 4 (top). A highly ($r^2 = 95.6\%$) significant relationship ($F_{1,8} = 174.6, p < 10^{-6}$) was found between the two modes of estimating the assay endpoint, and the regression slope of 0.934 did not differ significantly from 1.000 ($t_{8} = -0.95, p > 0.10$). The CVs for mean growth rates obtained by microscopy ranged from 4 to 11% (mean = 6.4%), and those for growth rates determined by spectrophotometry from 1 to 13% (mean = 6.2%). Neither a significant correlation was found between them ($t_{8} = -0.018, p = 0.96$), nor the two groups of CVs differed significantly from each other ($t_{18} = -0.46, p = 0.65$).

Evaluation I: Assay potential for assessments in lotic systems

Mean current velocity during the 10-d experiment was 1.30 m/s at site R7. A good performance of the in situ assay system was observed at all study sites; assay apparatus and holding structure showed to be resistant, and the stability of the beads also was maintained after 10 d. The need to renew the nutrient
source increased with the rise in current velocity. For currents up to 0.03 m/s (R3 and R4) the nutrients did not dissolve within the 10-d exposure, while for currents faster than 0.5 m/s (R6 and R7), nutrients needed to be replaced after 5 d. After 5 d, both the CBEs and assay chambers presented some clogging by suspended sediment and organic particles. Also, some accumulation of flocculated particles inside the assay chambers was observed at all study sites.

**Evaluation II: In situ assay in a lentic system**

Table 1 presents water quality parameters monitored during the 21-d in situ assay in the lentic system. Although from day 3 onwards there was an increase in pH at both the impacted sites, mean pH values at S1 and S2 continued to be the lowest among all sites (means excluding day 0 values: 6.76 and 6.93, respectively). Such pH change was caused by the dynamics of the aquatic system; a decrease in the effluent discharge due to the end of a rainfall period has caused an upstream rise in pH. Mean conductivity values also were lower at the reference than at the impacted sites, except at site R2 most probably due to an elevated concentration of dissolved minerals. Mean minimum and maximum temperatures fluctuated between 8.6 and 15.1°C and between 10.9 and 16.7°C, respectively. Mean TSS values were comparable across sites, ranging from 2.3 (R2) to 4.7 mg/L (S2), though there was considerable variation within each site. Small differences in nutrient concentrations were found among sites, except for nitrate and silica, which tended to be highest at both impacted sites and at site R2. The concentration of chlorophyll-a was considerably higher at reference than at impacted sites. The highest metal concentrations were recorded at site S1 immediately followed by site S2. These concentrations were considerably higher than those at any of the reference sites, except for Cd and especially Ni.

From the factors susceptible of determining the growth of *C. vulgaris* at reference sites (extra LW+F chambers) throughout the 21-d exposure (minimum- and maximum-temperature, TSS, and exposure duration), only minimum-temperature and exposure duration contributed to significantly predict growth as the multiplication factor of the increase in cell density relatively to day 0 ($r^2 = 94.2\%$, $F_{2,9} = 76.1, p < 10^{-4}$). At the sites with a 21-d mean minimum-temperature of 8.6 (R1) and 9.1°C (R2), cell density increased by seven- and eight-fold after 21 d, respectively. For a mean 21-d minimum-temperature of 15.1°C (R3), an eight-fold increase in cell density occurred after 6 d and, according to the model, an increase by 16-fold was already achieved by day 8.

Exposure in control medium or in site water enriched with nutrients (LW+F) did not significantly influence the growth of *C. vulgaris* at any of the reference sites ($F_{1,6} = 0.076–0.92$, $p \geq 0.25$). Mean growth rates in controls ranged from 0.094 (R1) to 0.22 d⁻¹ (R3) and in LW+F from 0.089 (R1) to 0.21 d⁻¹ (R3), with CV ≤ 11% except for LW+F at R1 with a CV = 21%.

Mean growth rates of *C. vulgaris* after 21 d of exposure in control chambers (OECD medium) are plotted in Figure 5 against the 21-d mean minimum-temperature at each site. Analogous to the previous results, among the variables minimum- and maximum-temperature and TSS, only minimum-temperature significantly explained ($r^2 = 95.5\%$, $F_{1,3} = 64.1, p < 10^{-4}$) the differences in control growth across sites. The following model (Eqn. 1) explained the relationship between growth rate ($y$) and minimum-temperature ($x$; standard errors in parenthesis), within the range of 8.6 to 15.1°C.

\[
y = -0.076(\pm 0.028) + 0.019(\pm 0.0024)x
\]

Figure 6 shows *C. vulgaris* mean growth rates in treatments LW and LW+F as a percentage of control growth rates predicted by the Equation 1 model. After removing the minimum-temperature effect, significant growth rate differences were still found across sites, for both the LW and LW+F treatments ($F_{4,16} = 58.0–117.8, p < 10^{-5}$). Within each treatment, *C. vulgaris* grew significantly more (by at least 64%) at reference than at impacted sites, and no significant differences in growth were found within reference or impacted sites. Exposure using treatment LW+F instead of LW resulted in a significant increase in growth at sites R2, R3, and S2 ($F_{1,4} = 11.2–83.1, p < 0.025–10^{-5}$).

A highly ($r^2 = 90.4\%$) significant relationship ($F_{1,12} = 113.7, p < 10^{-7}$) was found between in situ growth rates determined by microscopy and spectrophotometry (Fig. 4, bottom), and a Student’s $t$ test indicated that the regression slope of 1.23 was not significantly different from 1.00 ($t_{12} = 2.15, p > 0.05$). The CVs of mean growth rates estimated by mi-
Table 1. Physico-chemical parameters and chlorophyll-α concentrations measured at the different reference (R1, R2, and R3) and impacted (S1 and S2) study sites throughout the 21-d in situ assay in the lentic system. Values are mean (range) for pH, conductivity (Cond), minimum and maximum temperature (Tmin, Tmax), total suspended solids (TSS), ammonia nitrogen (NH₃-N), nitrate nitrogen (NO₃-N), nitrite nitrogen (NO₂-N), reactive phosphorus (PO₄³⁻), silica (SiO₂), and chlorophyll-α (chl-α), and single values from composite samples for metals.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cond (μS/cm)</td>
<td>173 (168–184)</td>
<td>817 (775–849)</td>
<td>195 (192–201)</td>
<td>252 (210–421)</td>
<td>240 (210–354)</td>
</tr>
<tr>
<td>Tmin (°C)</td>
<td>8.6 (8.0–9.5)</td>
<td>9.1 (9.0–9.5)</td>
<td>15.1 (15.0–15.5)</td>
<td>12.2 (11.7–12.5)</td>
<td>12.8 (12.3–13.0)</td>
</tr>
<tr>
<td>Tmax (°C)</td>
<td>14.4 (13.0–16.0)</td>
<td>10.9 (10.0–12.0)</td>
<td>16.7 (16.0–17.0)</td>
<td>16.0 (15.8–16.1)</td>
<td>16.2 (16.0–16.5)</td>
</tr>
<tr>
<td>TSS (mg/L)</td>
<td>3.6 (2.1–4.8)</td>
<td>2.3 (0.7–3.8)</td>
<td>2.9 (1.9–5.7)</td>
<td>3.8 (2.4–5.5)</td>
<td>4.7 (1.6–8.9)</td>
</tr>
<tr>
<td>NH₃-N (mg/L)</td>
<td>0.33 (0.28–0.36)</td>
<td>0.31 (0.17–0.58)</td>
<td>0.28 (0.18–0.41)</td>
<td>0.36 (0.27–0.45)</td>
<td>0.36 (0.30–0.40)</td>
</tr>
<tr>
<td>NO₃-N (mg/L)</td>
<td>0.15 (0.00–0.20)</td>
<td>0.25 (0.10–0.50)</td>
<td>0.13 (0.10–0.20)</td>
<td>0.23 (0.00–0.50)</td>
<td>0.35 (0.20–0.60)</td>
</tr>
<tr>
<td>NO₂-N (mg/L)</td>
<td>0.002 (0.001–0.004)</td>
<td>0.002 (0.001–0.003)</td>
<td>0.011 (0.005–0.030)</td>
<td>0.005 (0.001–0.004)</td>
<td>0.003 (0.001–0.004)</td>
</tr>
<tr>
<td>PO₄³⁻ (mg/L)</td>
<td>0.11 (0.001–0.27)</td>
<td>0.15 (0.005–0.41)</td>
<td>0.15 (0.001–0.52)</td>
<td>0.03 (0.001–0.09)</td>
<td>0.14 (0.001–0.36)</td>
</tr>
<tr>
<td>SiO₂ (mg/L)</td>
<td>1.51 (1.11–1.96)</td>
<td>7.55 (7.03–7.98)</td>
<td>3.03 (2.59–3.80)</td>
<td>4.25 (2.95–6.97)</td>
<td>3.84 (3.17–5.46)</td>
</tr>
<tr>
<td>chl-α (g/m³)</td>
<td>0.20 (0.12–0.25)</td>
<td>0.35 (0.06–0.39)</td>
<td>0.26 (0.15–0.33)</td>
<td>0.06 (0.02–0.14)</td>
<td>0.08 (0.03–0.17)</td>
</tr>
<tr>
<td>Al (mg/L)</td>
<td>0.043</td>
<td>0.055</td>
<td>0.117</td>
<td>0.969</td>
<td>0.569</td>
</tr>
<tr>
<td>Cd (mg/L)</td>
<td>&lt;0.0005</td>
<td>0.002</td>
<td>&lt;0.0005</td>
<td>0.003</td>
<td>0.0024</td>
</tr>
<tr>
<td>Co (mg/L)</td>
<td>&lt;0.001</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>0.012</td>
<td>0.009</td>
</tr>
<tr>
<td>Cu (mg/L)</td>
<td>&lt;0.005</td>
<td>0.012</td>
<td>0.015</td>
<td>0.159</td>
<td>0.113</td>
</tr>
<tr>
<td>Ni (mg/L)</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>&lt;0.005</td>
<td>0.006</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Pb (mg/L)</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.004</td>
<td>0.003</td>
</tr>
<tr>
<td>Zn (mg/L)</td>
<td>&lt;0.01</td>
<td>0.036</td>
<td>0.464</td>
<td>0.347</td>
<td></td>
</tr>
</tbody>
</table>

a Parameter determined every 3 d (days 0, 3, 6, 9, 12, 15, 18, and 21).
b Parameter determined at days 0, 6, 12, and 18.
c < below the detection limit.
cells of *Chlorella vulgaris* means estimated by spectrophotometry from 2 to 37% (mean 15.1). No significant correlation ($r_{26} = −0.62, p = 0.54$) were found between the two groups of CVs.

**DISCUSSION**

**Selection of test species**

The two species of green algae investigated, *P. subcapitata* and *C. vulgaris*, were able to grow according to acceptability criteria adopted in ecotoxicological studies [12–14] when immobilized in beads of calcium alginate and cultured in media with essential nutrients (MBL and R+N). Apparently, their encapsulation in the alginate matrix and subsequent growth did not cause substantial reductions in light penetration or carbon dioxide and nutrient diffusion [21,24]. These results are in agreement with other studies showing the good growth performance of immobilized *P. subcapitata* [17] and *Chlorella* sp. cells [24]. Both *P. subcapitata* and *C. vulgaris* grew significantly more in nutrient enriched (R+N) than in plain reference water (R), suggesting that their immobilized cells also respond similarly to a change in water quality. These two species of green algae are among the most widely recommended for ecotoxicological purposes [12–14], mainly due to the ease with which they are maintained under laboratory conditions and their sensitivities to pollutants assessed. For *P. subcapitata*, a large database is already available on its response to a variety of chemicals and its relative sensitivity compared to other organisms [15]. However, whereas the latter has been found exclusively in oligothrophic and eutrophic waters in Central and Northern Europe and North America [34], *C. vulgaris* is known to be widespread in all water habitats and as the most frequently occurring species [35]. Given the good growth performance of immobilized *C. vulgaris* and its wider geographical distribution compared to *P. subcapitata*, *C. vulgaris* was selected to be included in the phytoplankton in situ assay. Yet, it should be underlined that to further improve the ecological relevance of an in situ assay, it might be advantageous to replace *C. vulgaris* by a local (or closely related surrogate) species, provided the latter grows well under laboratory conditions and when immobilized in alginate beads.

**Exposure with a nutrient source**

This study demonstrated the potential of the fertilizer as a nutrient source for in situ exposures. Despite significant differences in the growth of *C. vulgaris* between the control medium and the fertilizer, the latter was capable of sustaining algal growth according to acceptability criteria. Guaranteeing the existence of a similar composition of essential nutrients across study sites is important to allow the distinction of a toxic effect from that caused by poor nutrient concentrations. Adding nutrients to the test waters (in the same proportion as in control media) is an approach also recommended for laboratory algal assays when evaluating the potential hazard of effluents [14]. Furthermore, by eliminating nutrient limitations, the chances of algal growth to follow an exponential pattern and of detecting growth differences between reference and impacted sites are enhanced [9]. Another advantage is that algal growth at reference sites may reach recommended criteria [9,12,13] earlier than without nutrient enrichment, reducing the duration of the assay and increasing its cost-effectiveness. It can be argued that adding nutrients during exposure reduces the environmental relevance inherent to an in situ assay, because the algae are exposed to unrealistic nutrient levels. In addition, the sensitivity of microalgae to contaminants (particularly to metals) has been found to be influenced by the nutrient medium [36]. However, the proposal of a treatment supplementing the local water with nutrients does not intend to replace the deployment of algae exclusively to the site water, but to combine the information gained from both treatments.

**Assay duration—initial bead cell density vs temperature**

The growth of *C. vulgaris* was affected significantly by both the initial cell density in the beads and temperature. It generally increased with a rise in temperature and a decrease in initial bead cell density. However, whereas temperature explained 77% of the variation in growth, the initial bead cell density only explained a further 9% of the variation. Moreover,
the influence of initial bead cell density on growth was temperature-dependent (only significant at 15 and 20°C), whereas that of temperature occurred irrespective of the initial bead cell density. In a recent study on the influence of test conditions during algal assays, temperature also had a primary impact on the growth of control cultures, greater than light, nitrogen source, or pH [18]. The better growth of *C. vulgaris* in the low than in the high initial cell density beads was probably caused by constraints in the diffusion of light, nutrients, and carbon dioxide through the alginate matrix [21,24]. As expected, the influence of both initial bead cell density and temperature on *C. vulgaris* growth rate determined the experimental duration needed for the adopted cell density criterion to be achieved. The time required for an eight- and 16-fold increase in population density to be achieved decreased as temperature increased, and within each temperature generally was the same for the low and high initial cell density beads. Thus, results from this study strongly suggest that temperature will be a key factor determining the duration of an algal in situ assay. Given the small differences in algal growth and ideal assay duration observed between the low and high initial bead cell densities, and the fact that cell density estimates are more accurately performed on concentrated samples, an initial bead cell density of approximately 10^5 cells/ml of alginate was selected for the algal in situ assay.

**Assay endpoint estimation—microscopy vs spectrophotometry**

Microscopic cell counting is a simple, inexpensive, and reliable method for estimating microalgal growth rates that allows the direct examination of the condition of the cells, and the distinction between algal cells and cell debris. However, microscopy is time-consuming, especially when algal assays are part of routine toxicity evaluations. On the contrary, estimating algal growth through spectrophotometry can be carried out easily and in a short period of time, enabling a huge increase in the number of assays that can be performed. Results from the last laboratory experiment demonstrated that spectrophotometry is a suitable methodology to replace microscopy in the estimation of immobilized *C. vulgaris* growth rates, as a very good significant relationship \( r^2 = 96\% \) was found between both methods of assay endpoint estimation. Coefficients of variation showed that estimating growth by microscopy or spectrophotometry resulted in a similar variation. The absence of a significant correlation between the CVs of the two modes of determining growth suggests that the variability was associated with fluctuations in experimental variables and not with the method itself.

**Assay potential for assessments in lotic systems**

This study demonstrated that the developed assay can be deployed successfully in lotic systems. The physical resistance of the test system was verified for currents up to 1.3 m/s. Furthermore, the visual inspection of the beads strongly suggested that bead disruption with the consequent loss of algal cells to the surrounding medium [37] did not take place, and hence that bead stability was maintained at all study sites. After 10 d at currents up to 0.03 m/s, most of the fertilizer was still present in the chambers. Conversely, a great amount of fertilizer (>50%) was dissolved after 5 d at currents faster than 0.5 m/s. For such currents, the renewal frequency of the fertilizer should be increased. But, at currents higher or equal to 1.3 m/s, the extremely fast dissolution of the fertilizer (<6 h) makes its use impractical. To overcome this limitation, a possible solution might be to use a nutrient source with a slow dissolution rate. The need to clean the test system was evident from the extension of mesh fouling and accumulation of flocculated particles inside the chambers. Because an increase of the mesh sizes is hampered by the need to prevent the entrance of indigenous organisms, the fouling and accumulation of fines within cages can only be diminished by increasing considerably the cleaning frequency of the assay apparatus. However, such a decision should take into consideration the increase in labor that is involved. In the present study, only a cleaning frequency of once every 3 d was feasible, due to the long distance between the study area and the laboratory.

**In situ assay in a lentic system**

The deployment of the algal assay in the lentic aquatic system impacted by acid mine drainage further demonstrated the suitability of its chambers and procedures for in situ toxicity evaluations. Results from the extra assay chambers deployed at the three reference sites to periodically check for algal growth confirmed the value of such approach. From the factors assumed to be limiting, only minimum-temperature significantly determined the factor of increase in *C. vulgaris* cell density throughout the assay. At 15°C the ideal assay duration was estimated to be approximately 8 d, whereas after 21 d at 8 to 9°C the cell density in the beads increased by merely eight-fold. The absence of an effect of the total suspended solids probably was related to the small differences registered across sites, and also to the fact that the assay chambers were positioned just below the water surface. In this study, the influence of temperature on algal growth was observed to be similar in the laboratory and in the field. These results suggest that the dependence of algal growth on temperature can be determined in the laboratory before deploying the assay, to help predict the ideal assay duration. In this way, the number of checks can be reduced, increasing the cost-efficiency of the assay. Extending the assay well beyond the recommended increase in population density may cause constraints in the diffusion of nutrients, carbon dioxide, and light inside the alginate matrix [21,24]. Besides, the exposure of alginate beads during long periods also may lead to some degradation by microorganisms [17] or chelating agents [37]. In accordance to the laboratory results, in the situ assay confirmed the suitability of the fertilizer to supply nutrients during exposure, and thus the validity of using treatment LW + F to check for algal growth at the reference sites throughout the assay. Exposure at field sites in control medium or in site water enriched with fertilizer resulted in comparable *C. vulgaris* growth rates. Results from this assay also confirmed the suitability of spectrophotometry to estimate in situ growth. Not only a very good agreement \( r^2 = 90\% \) was found between growth rates estimated by microscopy and spectrophotometry, but both estimations led to a similar variation (CVs).

The growth of *C. vulgaris* in control chambers was in close agreement with previous results. It was significantly influenced by the minimum-temperature, but not by the maximum-temperature and total suspended solids. Because minimum-temperature differences across sites explained 95% of the among-site variation in control growth, the model expressing the relationship between growth and minimum-temperature was used to remove the minimum-temperature effect at each site, and ascribe among-site differences in growth to the sole impact of the mine effluent. Even after removing the temperature ef-
fect, a significant inhibition of *C. vulgaris* growth (≥64%) was found at the two impacted sites, whether the alga was exposed without (LW) or with (LW+F) a supply of nutrients. As nutrient levels in treatment LW+F were not limiting for algal growth, the mine effluent should have been the reason for the inhibition of growth observed at sites S1 and S2 with both treatments. Although the addition of fertilizer slightly decreased the toxic impact of the mine effluent at site S2, *C. vulgaris* growth at the two impacted sites still was comparable. Adding nutrients caused a significant increase in growth also at sites R2 and R3 but not at site R1. Because temperature was more limiting for algal growth at site R1 than at sites R2 and R3, the addition of nutrients (OECD/fertilizer) probably was not effective to cause a marked increase on algal growth at R1. Accordingly, relative growth at this site was the highest among the three reference sites. As pointed out by DeWitt et al. [1], the removal of effects of controlled factors to identify other treatment effects in bioassays should be applied with caution because controlled and treatment effects may interact. Yet, the marked toxic impact of the mine effluent observed in this study rules out the occurrence of any important interaction effect between environmental variables and toxicity. At moderately impacted sites, the identification of the factors causing an inhibition on algal growth probably would be more complex and thus more decisively call for an integrative study [2,6,8].

The ecological impact caused by acid mine drainage mainly is associated with low pH and high concentrations of heavy metals [31]. It was indeed at the two sites with lowest pH and highest metal concentrations (S1 and S2) that the growth of *C. vulgaris* was inhibited. However, except at deployment, the pH at these sites was weakly acidic and within the levels considered favorable for algal growth [13,18,31]. Metal toxicity generally is due to the activity of the free metal ion, with metal speciation and bioavailability being affected markedly by key water variables such as pH, hardness, and complexing agents [31,38]. It is expected that, at pH values of 6.47 (S1) and 6.66 (S2), Al is present chiefly in the form of soluble complexes, whereas Pb and Cu, but especially Co, Ni, Cd, and Zn, predominate in their free metal ion form [31,38]. Yet, levels of total Pb, Co, Ni, Cd, and Zn in the water were one to two orders of magnitude lower, and those of Cu slightly lower, than the concentrations reported to cause a 50% inhibition in the growth of *C. vulgaris* over a 24- to 96-h exposure [30–32,39]. It is possible that the apparent high metal toxicity observed in the present study may be due to the longer exposure period of the algae (21 d) compared to a maximum exposure of 96 h in the previous studies. However, toxic responses to a metal mixture have been demonstrated to be difficult to interpret because they are not predictable from the effects of the individual metals; metal interactions can produce synergistic or antagonistic toxic effects [32,38,40]. Thus, another possible explanation for the pronounced growth inhibition of *C. vulgaris* observed in this study, paralleled by a sharp decrease in chlorophyll-α, would be the occurrence of a synergistic interaction between some metals (e.g., Cu and Cd, and Cu and Co [32,40]). The accumulation of flocculated particles within the assay chambers also could be associated with the toxic response observed. Because metals exist in water in equilibrium between their ionic, dissolved, and complexed forms [31,38], a change in a water parameter favoring metal desorption (e.g., a pH decrease) would lead to higher concentrations of the free metal ion inside than outside the chambers. Due to the complexities of metal interactions and their dependence on water quality parameters, a weak correlation between total/dissolved metal levels and toxicity has been observed in various studies on the impact assessment of acid mine drainage [6,8,26,27].

In general, the phytoplankton in situ assay developed here was demonstrated to be an ecologically relevant and cost-effective tool suitable to routinely assess water quality at impacted aquatic systems. To date, only a few studies on the use of phytoplankton cells immobilized in alginate gel for in situ toxicity evaluations have been published [3,16,17]. A similar test system was proposed by Moreira dos Santos et al. [3] for estuarine environments, but the present design maximizes practicability and simplicity at deployment and light exposition. Compared with the in situ assays proposed specially for lotic systems by Twist et al. [16] and Faafeng et al. [17], the present developed system is made of inexpensive materials (estimated cost of a CBE and outer chamber was ~12 U.S. dollars), and its simple design allows the rapid and easy deployment of the assay directly to the continuously changing field conditions. In particular, the need to estimate algal growth periodically in the laboratory and then return the alginate films to the field makes the assay proposed by Twist et al. [16] somewhat demanding. Overall, this novel assay presents two major assets. First, a high within-assay response reproducibility because the low costs coupled with the high level of simplicity of the test system allowed the establishment of sub-replicates within replicates. Second, the inclusion of strategies to distinguish contaminant effects from effects associated with differences in environmental variables across study sites; either similar conditions were supplied at all sites (e.g., nutrient levels), or a methodology to account for differences in environmental variables (e.g., temperature) between sites was applied. The major limitations of the proposed assay are concerned with its application to some environments. If used in closed and small aquatic systems (e.g., ponds), the nutrient inputs to the water might contribute to eutrophication. Also, in systems characterized by high amounts of suspended particles, extensive mesh fouling can lead to severe restrictions in water flow and light penetration. Furthermore, its application in fast-flowing waters is limited by the rapid dissolution of the fertilizer.

**Acknowledgement**—This research was partially funded by Fundação para a Ciência e a Tecnologia (Portugal) (postdoctoral grant to the first author, Sensitivity and Ecological Relevance of New Methods for Risk Assessment Project, reference PRAXIS/PCNA/C/BIA/0157/96, and Pursuing Investigative Pathways in Environmental and Science Education Project, reference POCTI/CEC/34891/99) and by the European Union (Integrated Assessment Tools to Gauge Local Functional Status within Freshwater Ecosystems Project, reference EVK1-1999-00005). Thanks also are due to André Moutinho for help in using the CorelDRAW® 10.0 Software (Ottawa, ON, Canada), and to António M. Santos for going through the text.

**REFERENCES**


