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Influence of pH on the survival of *Dictyosphaerium chlorelloides* populations living in aquatic environments highly contaminated with chromium

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ABSTRACT

The accommodation of photosynthetic organisms to adverse conditions, such as pH changes in the aquatic environment, and their response to aquatic pollutants is essential to develop future biosensors. The present study reports the ability of both Cr(VI)-sensitive and tolerant *Dyctiosphaerium chlorelloides* strains to live in aqueous solutions highly contaminated with hexavalent chromium under varying ranges of pH, by the determination of chromium toxic effects on these strains. Studies of cell growth, photosynthetic quantum yield and gross photosynthesis rate show that both *D. chlorelloides* strains are able to survive in alkaline and moderately acidified (pH 4.25) aquatic environments. Below this pH value cell populations from both strains exposed for short periods of time to Cr(VI) showed alterations in the three parameters studied. There were no significant differences comparing the response of both strains at pH change in the culture medium. However, Cr(VI)-tolerant strain exhibits a better fit to maintain cell growth than Cr(VI)-sensitive strain when both were subjected to pH 4.25 in the culture medium. The absence of significant differences in photosynthetic activity results for both strains suggests that the lower sensitivity exhibited by Cr(VI)-tolerant strain would be due to cellular morphological changes rather than changes in cellular activity.

1. Introduction

The presence of heavy metal ions in surface water continues to be the most pervasive environmental issues of present time (Postma et al., 2006; Zhou et al., 2008). Chromium is one of the contaminants, which exists in hexavalent and trivalent forms. In unpolluted freshwater environments, dissolved chromium concentrations can reach values close to 0.4 μM (Ochieng et al., 2008). It has been detected that in the contaminated areas, water may contain dissolved chromium concentrations as high as 2 μM (Bobrowski et al., 2004).

Hexavalent form is more toxic (Smith and Lec 1972) than trivalent and requires more concern. Chromium salts are used extensively in metal finishing industries such as electroplating, leather tanning, in pigments, in fungicides and in wood preservatives. As a result of the widespread use in numerous industrial processes, chromium is a pollutant of air, soil and water (Cervantes et al., 2001).

A wide variety of microorganisms such as bacteria, yeast, algae, protozoa and fungi are found in waters receiving industrial effluents. Many of the microorganisms show adaptation to the toxic materials constantly released into their environment. They have developed strategies to resist, tolerate, metabolize, and to detoxify these toxic substances (Parsek et al., 1995). Multiple metal resistant algae have been reported in a number of studies (Haq and Shakoori, 1998; Rehman and Shakoori, 2001; Nishikawa and Tominaga, 2001).

In the presence of toxic metals such as chromium, extra- or intracellular detoxification processes can occur in freshwater unicellular green algae. The processes may depend on constitutive and/or adaptive mechanisms and involve different compounds (Pinto et al., 2003; Sanità et al., 2003). Based on these processes, some authors have shown that the tolerance to Cr(VI) was heritable since the Cr(VI)-tolerant strain was able to grow in the presence of high Cr (VI) concentrations even after prolonged culturing in Cr(VI)-free medium (Corradi et al., 1995a, 1995b; D'ors et al., 2010).

Survival in extreme environments often involves significant changes in pH. Most phytoplankton isolates originating from alkaline lakes reach their optimum growth rate and photosynthetic capacity at a neutral or alkaline pH and are unable to survive in acidic conditions. In contrast, a limited number of algal species grow and photosynthesize in acid habitats (Gerloff-Elias et al., 2005). Whether a species can grow at neutral pH or not, defines it as either an acid-tolerant or acidophilic species (Gross, 2000).

The pH values can fluctuate between 2 and 12 in lakes and rivers (Wetzel, 2001), in close relation to the geology and

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hydrology of their drainage basins. Larger pH fluctuations, up to 2 pH units, occur in lakes where the buffering capacity of the carbonate system is less efficient, in highly productive small water bodies, and in the littoral zones of shallow lakes with intense primary production (Talling, 1976).

Although there is some evidence for species-specific pH tolerance of freshwater microalgal species, primarily originating from cursory field measurements (Costas et al., 2007; López-Rodas et al., 2008), an experimental laboratory investigation of the pH reaction norm of strains adapted to these environmental conditions is still lacking. There are multiple evidences in the bibliography indicating as pH fluctuations by 1–2 units imply 10- to 100-fold changes in free hydrogen ion activity, and it is well known that hydrogen ion concentration gradients affect many transport processes across cellular membranes and metabolic functions in the cytoplasm and cellular organelles (Prescott et al., 2002). Further, pH has a strong impact on the solubility, bioavailability, and toxicity of different aquatic contaminants, including heavy metals (Anderson, 1988; Wetzel, 2001). Rapid adaptation of microalgae to environmental changes resulting from chromium pollution has been shown (Sánchez-Fortún et al., 2009), unfortunately the evolution of these chromium tolerant microalgae subsequent to environmental change is insufficiently understood.

Photosynthesis is considered in most aquatic toxicity studies. For studies of phytoplankton photosynthesis, three components are usually defined: gross photosynthesis (P_g), respiration (R) and net photosynthesis (P_n), where P_n is the difference between P_g and R. Ryther (1956) argued that P_n is of the most ecological significance because it represents the tangible quantity of organic matter added to the environment. Odum (1983) suggests that the ratios of R to P_g and of R to biomass are important measures of ecosystem order, with well-ordered, diverse ecosystems having lower ratios. Therefore, having a means of measuring both net photosynthesis and respiration using the same method provides a more complete means of evaluating ecosystem structure and dynamics.

Thus, the possible adverse effect on two strains of *Dictyosphaerium chlorelloides*, sensitive and tolerant to hexavalent chromium in the medium, was studied under different pH values. The tolerance of these microalgal strains for their growth and photosynthetic performance in an extreme environment with these changes in pH, was analyzed by using an experimental model.

2. Methods

2.1. Chemicals

Chromium(VI) oxide (CrO_3), was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). This compound was dissolved in distilled water. In all assays, the working solutions were freshly prepared each time.

2.2. Experimental organisms

Hexavalent chromium sensitive (Dc1M^{wt}) and tolerant (Dc1M$^{Cr(VI)R25}$) strains of *Dyctiosphaerium chlorelloides* (Nauman) Komárek & Perman, from the algal culture collection of the Environmental Toxicology Laboratory (Toxicology and Pharmacology Department, UCM, Madrid, Spain), were used in these assays. Dc1M^{wt} strain grew axenically in culture flasks (Greiner, Bio-One, Longwood, NJ, USA) with 20 ml of BG-11 medium (Sigma, Aldrich Chemie, Taufkirchen, Germany), while Dc1M$^{Cr(VI)R25}$ strain was cultured in BG-11 medium supplied with CrO_3 0.25 mM. Dc1M$^{Cr(VI)R25}$ strain was obtained from Dc1M^{wt} by selection of spontaneous mutants that showed an increased tolerance to Cr(VI).

Both strains were maintained at 20 °C and a photon irradiance of 60 μmol m^{-2} s^{-1} over the waveband 400–700 nm, in a 16:8 h light-dark photoperiod. Cells were maintained in mid-log exponential growth by serial transfers of one-cell inocula to fresh medium once a fortnight. Prior to the experiments, the cultures were recloned (by isolating a single cell) to avoid including any previous spontaneous mutants accumulated in the cultures.

2.3. Toxicity tests

To determine the inhibition of algal growth, effective quantum yield (Φ_{PSII}) and oxygen evolution, toxicity tests were performed in 5 mL polystyrene sterile culture tubes (Sarstedt Co., Nümbrecht, Germany) filled with BG-11 medium. Previous studies determined the suitability in the use of polystyrene sterile culture tubes for these toxicity assays, assuring that chemicals and cells do not adhere to the wall tubes (Costas et al., 2001; García-Villada et al., 2002). The water used for media preparation was of ultrapure quality, distilled by means of Milli-Q device (Millipore, Bedford, MA, USA).

To determine the stress induced in both *D. chlorelloides* strains by the pH variation in the medium, HCl and NaOH were added to culture medium in the appropriate amount to achieve final nominal pH levels of 3, 4, 5, 6, 7, 8, 9, 10 and 11. Through these experiments, minimum pH value of 4.25, which did not induce any effect on both strains, was established. This pH value together with the pH value of 7.5 (pH of medium BG-11) will be used in the following toxicity tests.

To determine the toxic effect induced by Cr(VI) in both *D. chlorelloides* strains, preliminary toxicity tests were performed to define the range of concentrations that included 0% and 100% inhibition. The results obtained established a concentration range of 2–100 μM and 0.1–10 mM to Dc1M^{wt} and Dc1M$^{Cr(VI)R25}$, respectively. In order to confirm that Cr(VI) concentrations were maintained throughout the exposure time, culture medium samples were analyzed to determine Cr(VI) concentrations at 24, 48 and 72 h time intervals. Cr(VI) concentrations were determined colorimetrically using a spectrophotometer (Merck Spectroquant® NOVA 400, NJ, USA) at 540 nm by reaction with 1,5-diphenylcarbazide in acid solution (APHA, 1992).

Each assay was repeated eight times ($n=8$). Both control and test tubes were inoculated with 10^4 cells mL^{-1} as initial concentration. All the cultures (control and treatments) were incubated for 72 h at 20 °C in a thermostatically controlled chamber (Velp Scientifica, Usmate, Italy) at 60 μmol m^{-2} s^{-1} to ensure exponential algal growth. Every 24 h the algal density was quantified under the light microscope. The concentration causing 50% growth inhibition of algae was determined by using light microscope and Neubauer's chamber (Brand, Wertheim, Germany).

The quantity causing 50% inhibition of photosynthetic yield (Φ_{PSII}) was obtained by means of the dual-channel PAM chlorophyll fluorometer (ToxY-PAM, Heinz Walz GmbH, Germany). The ToxY-PAM dual-channel yield analyzer obtains highly sensitive measurements of effective quantum yield of the algae pigment system II centers via assessment of the chlorophyll fluorescence yield and the saturation pulse method (Schreiber et al., 2002).

Gross photosynthesis rate (P_g) was estimated from the formula

$$P_g = P_n + R$$

where P_g corresponding to the oxygen production rate of photosystem II, R (respiration) corresponding to the process by which in the presence of light microalgal cells consume oxygen and releases carbon dioxide, and P_n (net photosynthesis rate) is defined as the difference between P_g and R. The oxygen values were obtained employing a Clark-type electrode. Dissolved O_2 was measured in a 1 ml reaction chamber from a Chlorolab 2 System (Hansatech, Norfolk, UK). Chlorolab 2 provides a system for the study of respiration and photosynthesis from liquid samples under automated illumination from red (660 nm) LED light. In the toxicity assays, measurements were taken at 20 °C and photosynthetic photon flux density (PPFD) of 975 μmol m^{-2} s^{-1}.

These parameters (growth rate, Φ_{PSII} activity and P_g rate) were used as toxic endpoints and they were expressed as 72-h median inhibitory concentration [$IC_{50(72)}$].

2.4. Data analysis

The 72 h EC_{50} values were calculated according to the 'area under the curve' method prescribed by the ISO (ISO 8692, 2012). EC_{50}-values were determined by nonlinear regression analysis, and all the results are presented as mean ± SD. The Φ_{PSII} activity was monitored on the software package ToxyWin v1.14 (Heinz Walz GmbH, Germany), and the results are presented as mean ± SD of inhibition percentage with regard to control. Oxygen measurements obtained both in darkness and in light conditions, were exported to a computerized chart recorder (Oxigraph v1.01, Hansatech, Norfolk, UK). Statistical analysis was performed using the computer software package GraphPad Prism v5.0 (Graph-Pad Software Inc., USA). The experimental data was analyzed by the one-way analysis of variance (ANOVA) and the differences were considered significant at $P<0.05$.

3. Results

3.1. pH stress

After 72 h of exposure, cell populations of both *D. chlorelloides* strains exposed at pH values above 7.5 (8–11) had no significant