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Metals and linear alkylbenzene sulphonate as inhibitors of the algae *Pseudokirchneriella subcapitata* acid phosphatase activity

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Abstract Sewage sludge applied to soils as a fertilizer often contains metals and linear alkylbenzene sulphonate (LAS) as contaminants. These pollutants can be transported to the aquatic environment where they can alter the phosphatase activity in living organisms. The acid phosphatase of algae plays important roles in metabolism such as decomposing organic phosphate into free phosphate and autophagic digestive processes. The order of in vitro inhibition of *Pseudokirchneriella subcapitata* acid phosphatase at the highest concentration tested was LAS > Hg^{2+} = Al^{3+} > Se^{4+} = Pb^{2+} > Cd^{2+}. A non-competitive inhibition mechanism was obtained for Hg^{2+} (K_i = 0.040 mM) and a competitive inhibition for LAS (K_i = 0.007 mM). In vivo studies with treated algae cultures showed that the inhibition of specific activity was observed in algae exposed during 7 days, in contrast to short term (24 h) treatments with both these chemicals. Our results suggest that the inhibition parameters in vitro did not markedly differ between the two chemicals. On the other hand, in vivo evaluations showed strong differences between both pollutants regarding the concentration values and the degree of response.

Keywords Mercury · Toxicity · Enzyme · Pollutant · *Selenastrum capricornutum* · Phytoplankton

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Introduction

Sewage sludge applied to agricultural soils as a fertilizer often contains considerable amounts of inorganic (Srikanth et al. 1992; Mamais et al. 2000) and organic (Erhardt and Prüeß 2001) contaminants. The transport of these chemicals from treated soils to water bodies, followed by the adverse effects to the biota, is a result of two mechanisms: waterborne runoff (Rand and Petrocelli 1985) and lixiviation (James 1977; WHO 1996). Some of these inorganic pollutants such as Al^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} which are not essential for cell viability and even in very low amounts can promote biochemical impairments. For example, Hg^{2+} and Cd^{2+} inhibit algal β-D-galactosidase (Peterson and Stauber 1996) and algal β-glucosidase (Obst 1988), as well as fish phosphatases (El Demerdash and Elagamy 1999; Gill et al. 1991) and mollusc acetylcholinesterase (Machreki-Ajmi et al. 2008). The activities of glucose-6-phosphate dehydrogenase (Kong and Chen 1995) and phosphatases (Rai et al. 1998) were decreased by Al^{3+} in green algae species. Inhibitions of acetylcholinesterase (Martinez-Tabche et al. 1988) and urease (Obst 1988), by Pb^{2+}, respectively, in aquatic invertebrates and river phytoplankton have been reported.

Other metals such as Se^{4+} are essential nutrients for living cells since they act as cofactors for enzymes. In particular, Se^{4+} is an essential component of the glutathione peroxidase (Barceloux 1999). However, higher concentrations of this metal are toxic to cells and are able to inhibit enzymes such as hepatic drug-metabolizing system enzymes (Ishikawa et al. 1992) and hepatic delta-aminolevulinate dehydratase (Farina et al. 2003) and UDP-*N*-acetylglucosamine kinase (Zeitler et al. 1992). Copper, another essential metal, is able to change the kinetic parameters of phosphatases in crude extracts from honeybees (Bounias et al. 1996).

The anionic surfactant linear alkylbenzene sulphonate (LAS) can be found in very high concentrations in most sewage sludges (Jensen 1999). Fernandez et al. (1995) studied the adsorption of LAS by algae and demonstrated initial adsorption rates decrease by increasing the pH values. The effects of LAS in biochemical processes have been associated with decreases in enzyme activity, as observed for fish succinic dehydrogenase (Mohan and Verma 1981), fish phosphatase (Trivedi et al. 2001), plant phosphatase (Tanaka et al. 1975) and microbial lipase (Helisto and Korpela 1998).

Pseudokirchneriella subcapitata (formely *Selenastrum capricornutum*), an unicellular chlorophyceae (green) alga present in the aquatic and terrestrial compartments (Keddy et al. 1995) has been widely used in studies of agriculture pollutants effects (Jonsson et al. 1998; Munkegaard et al. 2008) and recommended by regulatory national (Gherardi-Goldstein et al. 1990; Jonsson and Maia 1999) and international (OECD 1984) agencies as a test organism.

Algal acid phosphatase plays important roles in metabolism such as decomposing organic phosphates into free phosphates and organic compounds. Several other functions have been attributed to algal acid phosphatases such as participation in autophagic digestive processes, hydrolysis of phospholipid materials (Cooper et al. 1974), fertilization (breakdown of plasmalemma and absorption of flagella) (Braten 1975), releasing of inorganic phosphate from the extracellular medium (Sommer and Blum 1965) recycling of inorganic phosphate for its reassimilation (Theodorou et al. 1991), endomembrane recycling (Domozych 1989) and spore differentiation (Tsekos and Schnepf 1991).

In this work, based on the frequent presence in sewage sludge and the toxicity in aquatic organisms, we studied and compared the inhibitor effect of metals and LAS on acid phosphatase extracted from *Pseudokirchneriella subcapitata*. Moreover, we focused our investigation on two contaminants, Hg^{2+} and LAS, by determining enzymatic parameters of their inhibition and evaluating the phosphatase activity in vivo after exposure to these chemicals.

Materials and methods

Materials

p-Nitrophenylphosphate (*p*NPP) was obtained from Sigma Chemical Co. Stock solutions of $HgCl_2$, $Pb(NO_3)_2$, $CdCl_2$, $Al_2(SO_4)_3$, $Na_2SeO_3 \cdot 5H_2O$ and linear alkylbenzene-sulfonic acid sodium salt (LAS) were prepared in Milli-Q water. In vivo assays, the pollutants were added to a medium for algae culture as recommended by OECD (OECD 1984). All reagents were AR grade.

Organisms and growth conditions

The unicellular green alga *P subcapitata* was maintained in axenic culture and subcultured in an inorganic liquid medium prepared as recommended by OECD (OECD 1984). Cultures were grown in 250 mL flasks sealed with cotton bungs and containing 200 mL of sterilized medium. The flasks were incubated in a controlled temperature chamber (20 ± 2°C) under a continuous white fluorescent light of 3,000–4,000 lux and manually shaken twice a day. Every 40–60 days, a new culture was prepared by inoculating approximately 5×10^4 cells mL^{-1} (Jonsson and Aoyama 2007).

Harvesting and preparation of extracts

All centrifugation procedures were carried out at 4°C. Exponential phase organisms were harvested by centrifugation at 4,000 rpm. for 5 min in a Beckman J2-21 refrigerated centrifuge (rotor JA-20) and washed twice with 0.1 M sodium acetate buffer, pH 5.0.

The alga pellet was suspended in 0.1 M sodium acetate buffer (1:4 w/v) and the cell suspension was submitted to the following cell disruption procedures for phosphatase extraction: the sample was frozen at −20°C, thawed at room temperature and submitted to a probe sonication at 0°C (ice bath) for 50 s followed by a 20 s interval (1 cycle) with an amplitude of 70 (Vibra Cell, Sonics Materials Inc., 45 mm tipped probe). This procedure was repeated twice.

The resultant cell-disrupted suspension was centrifuged at 10,000 rpm. for 20 min and the supernatant fluid (extract) was used for the in vitro determination of acid phosphatase activity.

Assay of phosphatase activity

Acid phosphatase activity was routinely assayed at least in duplicate by incubating the enzyme with *p*NPP as substrate and measuring the *p*-nitrophenol (*p*NP) produced as previously described (Prazeres et al. 2004). The enzyme activity was determined in a final volume of 1 mL containing 0.1 M sodium acetate buffer (pH 5.0) and 10 mM substrate. After incubation for 40 min at 37°C, the reaction was terminated by the addition of 1 mL of 1 M NaOH.

For in vivo experiments, the samples were incubated during 60 min. The *p*NP released was measured at 405 nm in a UNICAM 8625 UV/VIS spectrophotometer. For the initial velocity [*V*] determination, the amount of *p*NP produced was calculated using a molar extinction coefficient of 18,300 M^{-1} cm^{-1} (Chaimovich and Nome 1970). Units (U) of enzymatic activity are defined as micromoles of *p*NP released per min. Specific activity is defined as units per mg of protein.

In vitro assays

Effect of concentration

Before the determinations of enzyme activity the enzyme was preincubated for 20 min at 37°C in the presence of three different concentrations of each pollutant. Enzyme activity was measured at several *p*NPP concentrations in the presence of 10 mM *p*NPP as substrate.

The compound concentration that promotes 50% of enzyme inhibition (IC_{50}) and its 95% confidence limits for mercury and LAS were calculated by adjusting the regression curve data (% activity vs. concentration) from enzyme activity at several doses of each pollutant.

Data were analyzed by Simples Regression and One Way ANOVA modules contained in the Statgraphics® Plus Version 2 software package. A p value < 0.05 was considered as significant.

Mercury and LAS inhibition constant (K_i) determination

Enzyme was assayed at eight concentrations (0.03–10.0 mM) of *p*-nitrophenyl phosphate (*p*NPP) as substrate in the absence or in the presence of the pollutants at three concentrations. Lineweaver–Burk plots were fitted in order to demonstrate the kind of inhibition (competitive or non-competitive).

The Enzyme Kinetics Module contained in the SigmaPlot 2001 software package (Sigmaplot, "Scientific Graphing Software, User's Manual," Jandel Scientific, San Rafael, CA, 1993) was used to calculate V_{max}, K_m and K_i values and its 95% confidence limits. The module fitted the experimental data by nonlinear regression to the Michaelis–Menten equation in its basic form (Eq. 1), with competitive inhibitor (Eq. 2) or with non-competitive inhibitor (Eq. 3):

$$V(S) = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

$$V(S,I) = \frac{V_{max}}{[1 + (K_m/S)(1 + I/K_i)]} \quad (2)$$

$$V(S,I) = \frac{V_{max}}{[(1 + I/K_i)(1 + K_m/S)]} \quad (3)$$

where V (mU mL^{-1}) is the velocity of the reaction at substrate concentration [S] (mM) and [I] (mM) is the concentration of the pollutant, V_{max} (mU mL^{-1}) is the maximum velocity and K_m (mM) is the Michaelis–Menten constant.

Mercury and LAS in vivo assays

Alga cell suspensions ($\sim 5 \times 10^6$ cells mL^{-1}) were exposed to each pollutant which was prepared in alga medium at three or five concentrations in glass flasks. The assays were performed with a control (no pollutant) by incubating at 20°C (2,000–3,000 lux) for 24 h (Tukaj and Aksmann 2007; Peterson and Stauber 1996) or 7 days (Jonsson et al. 2001; Tang et al. 1997). The cells suspensions were harvested by centrifugation, suspended in 3 mL sodium acetate buffer (pH 5.0) and submitted to freezing/thawing/sonication followed by centrifugation as described above. The enzyme activity and specific activity in the extract were expressed as U and U mg^{-1} protein, respectively.

Protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard.

The concentration that promoted 50% of decrease of the specific activity (EC_{50}) and its 95% confidence limits were calculated by adjusting a regression curve data from % specific activity versus pollutant concentration.

Data were analyzed by Simples Regression and One Way ANOVA modules contained in the Statgraphics® Plus Version 2 software package. A p value < 0.05 was considered as significant.

Results and discussion

We have previously demonstrated the in vitro effect of some agriculture pollutants and their joint action on *P. subcapitata* acid phosphatase (Jonsson and Aoyama 2007). In this work we describe in detail in vitro and in vivo inhibitions by metals and LAS on *P. subcapitata* acid phosphatase activity.

Enzyme inhibition and IC_{50} evaluations

Table 1 shows the effect of five metals and one organic compound on *p*NPP hydrolysis by the *P. subcapitata* acid phosphatase. Hg^{2+}, Se^{4+} and LAS demonstrated an inhibitory effect that was significant at the 95% confidence level. Al^{3+} promoted more than 50% inhibition that was significant only at the highest concentration tested. Pb^{2+} inhibited about 25% of activity at the highest concentration tested, although this effect was not statistically significant. A slight inhibition was observed by Cd^{2+} about 10% at 0.2 and 0.5 mM. The enzyme inhibition by 0.2 and 2 mM Se^{4+} did not vary significantly. Of these pollutants Hg^{2+} and LAS also exhibited appreciable inhibition at 0.2 mM, with 40 and 50% inhibition, respectively.

In the present investigation we observed that the pollutants Hg^{2+} and LAS induced in vitro modulation. A similar trend was noticed by Sugiura et al. (1981) who tested in vitro 30 organic and inorganic compounds on a plant acid phosphatase. Based on the in vitro results, the order of inhibition at the highest concentration tested was

Table 1 Activity of *P. subcapitata* acid phosphatase in the presence of several pollutants

Pollutant	Concentration (mM)	Relative activity (% ± SD)
Al^{3+}	0.02	106.50 ± 2.56
	0.2	94.89 ± 2.90
	2	45.48 ± 5.03*
Cd^{2+}	0.02	88.85 ± 3.46*
	0.2	92.42 ± 1.20*
	0.5	88.94 ± 2.34*
Hg^{2+}	0.02	80.27 ± 4.83*
	0.2	62.29 ± 5.80*
	2.0	43.66 ± 9.15*
Pb^{2+}	0.02	97.06 ± 4.35
	0.2	98.35 ± 1.97
	2	74.48 ± 6.23
Se^{4+}	0.02	89.56 ± 1.02*
	0.2	78.66 ± 3.20*
	2	76.92 ± 7.92*
LAS	0.02	89.41 ± 4.75*
	0.2	49.24 ± 1.19*
	2	13.95 ± 0.71*

The activity in the absence of these chemicals was considered as control (100%). Each value of relative activity was based on duplicate analyzes

* Significant decrease ($P < 0.05$) of activity in relation to the control

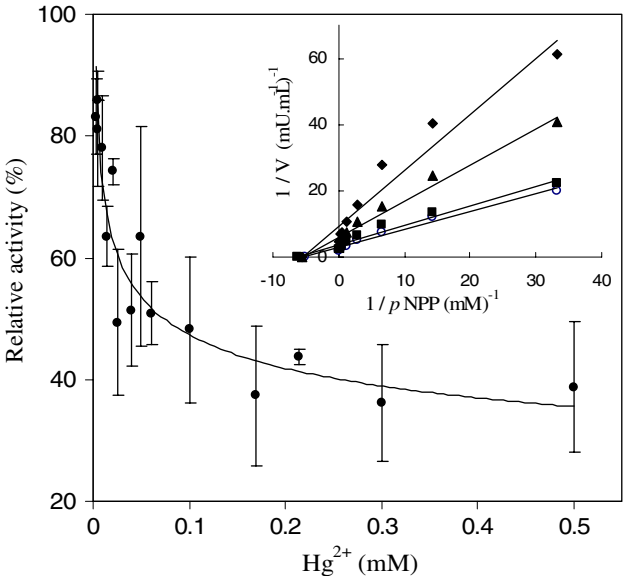

Fig. 1 Effect of Hg^{2+} concentration on *P. subcapitata* acid phosphatase activity. Enzyme activity was measured at several chemical concentrations in the presence of 10 mM *p*NPP as substrate. The system was incubated for 40 min at 37°C. Average values and standard deviations (*vertical bars*) from two experiments (analysis in duplicate) are shown. The activity in the absence of Hg^{2+} was considered as control (100%). *Inset*: Lineweaver–Burk plot. The enzyme activity was determined as described in Methods, at several substrate concentrations in the absence (○), and in the presence of 0.01 (■), 0.03 (▲) and 0.13 (◆) mM Hg^{2+}

$LAS > Hg^{2+} = Al^{3+} > Se^{4+} = Pb^{2+} > Cd^{2+}$. In contrast to these results, we observed an activating effect of Cu^{2+} on the acid phosphatase from *P. subcapitata* (results not shown). In analogy to these results, Mazorra et al. (2002) demonstrated that Hg^{2+} was the most potent inhibitor of alkaline and acid phosphatases extracted from mollusks.

In this work a study was made of the inhibition of *P. subcapitata* acid phosphatase by Hg^{2+} and LAS. In order to calculate the IC_{50} values for Hg^{2+} and LAS, the inhibition was performed at different concentrations of these pollutant agents with 10 mM *p*NPP as substrate. The obtained data were adjusted by linear regression that generated a logarithmic-*x* model curve ($y = a + b \ln x$) for Hg^{2+} and a reciprocal-*y* model curve ($y = 1/(a + bx)$) for LAS (Figs. 1, 2). The calculated IC_{50} and 95% confidence limits values based on the experiments with both pollutants using two different extract batches were 0.085 (0.064–0.117) and 0.289 (0.243–0.326) mM, respectively, for Hg^{2+} and LAS.

The degree of in vitro inhibition of phosphatases by Hg^{2+} and LAS has been described for some organisms. The estimated IC_{50} value of $HgCl_2$ for acid phosphatase from the alga *Ochromonas danica* in the 1–3 mM range (Patni and Aaronson 1974) was close to that determined in the present work. However, when compared with our results, this parameter was about 14 times lower for the metal inhibition of the enzyme extracted from gill mollusks (Mazorra et al. 2002). IC_{50} values of 0.5 and 0.025 mM were determined for fish liver (El Demerdash and Elagamy 1999) and crab (Chen et al. 2000) respectively.

Lineweaver–Burk plots showed that for Hg^{2+} the inhibition was non-competitive whereas LAS inhibited the enzyme in a competitive manner (Figs. 1, 2, insets).

The observed enzyme inhibition by Hg^{2+} or other heavy metals can be explained by their interactions with essential –SH groups. The presence of such groups in the active center of enzymes or in the stabilization of the quaternary structure are essential for enzyme activity (Van Assche and Clijsters 1990). Another mechanism of inhibition may be the deficiency of an essential metal in metalloproteins or metal–protein complexes, eventually, combined with substitution of the toxic metal for the deficient element (Omar 2002). Non-competitive inhibition correlated with Hg–SH interaction has been observed for other enzymes like fish creatine kinase (Araujo et al. 1996) horseradish peroxidase (Shubo et al. 2001) and fungal phosphomanose isomerase (Wells et al. 1994). Nevertheless, competitive (Araujo et al. 1996) and uncompetitive inhibitions (Chetty et al. 1990) for Hg^{2+} have been also reported.

The competitive inhibition properties of long chain anionic detergents like sodium dodecyl sulfate (SDS) with

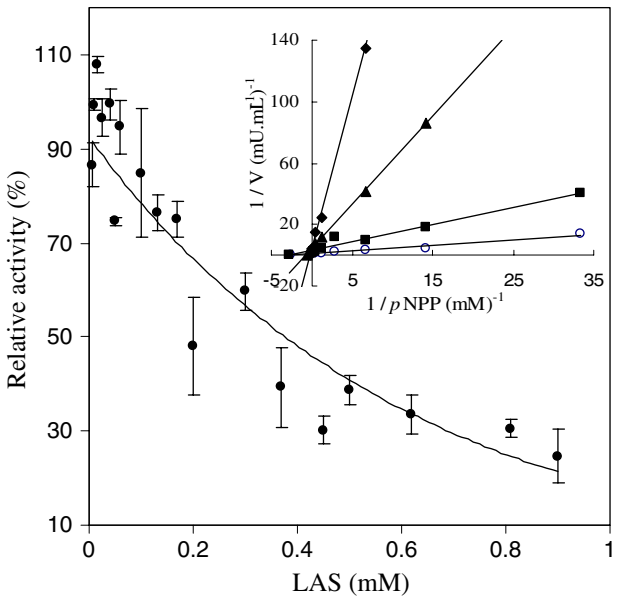

Fig. 2 Effect of LAS concentration on *P. subcapitata* acid phosphatase activity. Enzyme activity was measured at several chemical concentrations in the presence of 10 mM *p*NPP as substrate. The system was incubated for 40 min at 37°C. Average values and standard deviations (*vertical bars*) from two experiments (analysis in duplicate) are shown. The activity in the absence of LAS was considered as control (100%). *Inset*: Lineweaver–Burk plot. The enzyme activity was determined as described in "Methods", at several substrate concentrations in the absence (○) and in the presence of 0.1 (■), 0.2 (▲) and 0.3 (◆) mM LAS

enzymes such as mammalian lactate dehydrogenase and yeast glucose-6-phosphate dehydrogenase were observed by Vincenzini et al. (1982). According to these authors, both substrates pyruvate and glucose-6-phosphate, have either one negative charge or two charges concentrated on specific points of the molecule. Perhaps SDS, which possesses a negatively charged hydrophilic head, behaves as a competitive inhibitor. Similar considerations could be attributed to LAS inhibition since its negatively charged sulphonate group can compete with a negative charge on *p*NPP.

K_i determination for Hg^{2+} and LAS

The K_i values for Hg^{2+} and LAS were determined by nonlinear regression analysis from [*S*] versus *V* curves at three concentrations of the pollutant agents (Fig. 3). Calculated values of K_i (95% confidence limits) for LAS and Hg^{2+} were, respectively, 0.007 (0.004–0.009) and 0.040 (0.028–0.053) mM.

In comparison with ours results, a similar K_i value (36 μM) was calculated for the non-competitive inhibition by Hg^{2+} on alkaline phosphatase extracted from crab (Chen et al. 2000).

As described previously, the IC_{50} value determined for Hg^{2+} was about three times lower than that obtained for LAS. However, both compounds exhibited affinity for the enzyme in a close order of magnitude as demonstrated by the calculated K_i values. The determined K_i values for Hg^{2+} and LAS are, respectively, 10 and 59 times lower than the calculated K_m value in the absence of inhibitors (0.416 mM) indicating higher enzyme affinity for the pollutants compared to the substrate.

In vivo effect of Hg^{2+} and LAS on *P. subcapitata*

In the short term exposure study, cultures of *P. subcapitata* growing in OECD medium were supplemented with $HgCl_2$ to a final concentration range of 10–242 μM, according to data of a preliminary 24 h experiment. Figure 4a showed a significant decrease on enzymatic activity which was concentration-dependent. In a concomitant manner with this activity decrease, a significant diminution of protein content at 23 μM and higher concentrations was observed. An increase in the specific activity was observed at 23–242 μM, nevertheless it was statistically significant only at the highest tested concentration.

We also studied the in vivo effect of Hg^{2+} by the algae cultures exposed during 7 days at three concentrations of the metal (Fig. 4b). A 40 and 70% diminution of enzyme activity was shown for the lowest and the highest Hg^{2+} concentration tested, respectively. The data allowed us to calculate an EC_{50} value of 7.96 (5.00–9.72) μM for such inhibition.

A marginal decrease in protein content in relation to the control was observed for all concentrations tested, nevertheless it was only significant at 5 and 15 μM. Because this slight protein diminution was accompanied by a strong inhibition of the enzyme activity, a marked decrease in the specific activity was observed at 10 and 15 μM. The EC_{50} calculated for this parameter was equivalent to 12.63 (9.78–17.61) μM.

During 24 h exposure, an increment of enzyme activity dependent on increasing LAS concentration was shown in Fig. 5a. This effect was significant at 10 mM LAS and was also observed for the protein content in the extract. No significant variation in the specific activity from controls was noted for all the LAS concentrations tested.

A non-significant increase of enzyme activity was observed at 0.02 mM LAS after 7 days exposure (Fig. 5b). On the other hand, a significant depression on enzyme activity was observed at 2 mM. No alteration in protein content was evidenced at the LAS concentrations tested. Therefore, a diminution on specific activity was observed at the highest LAS concentration.

In contrast to the results obtained in vitro experiments, very different patterns of effects between Hg^{2+} and LAS

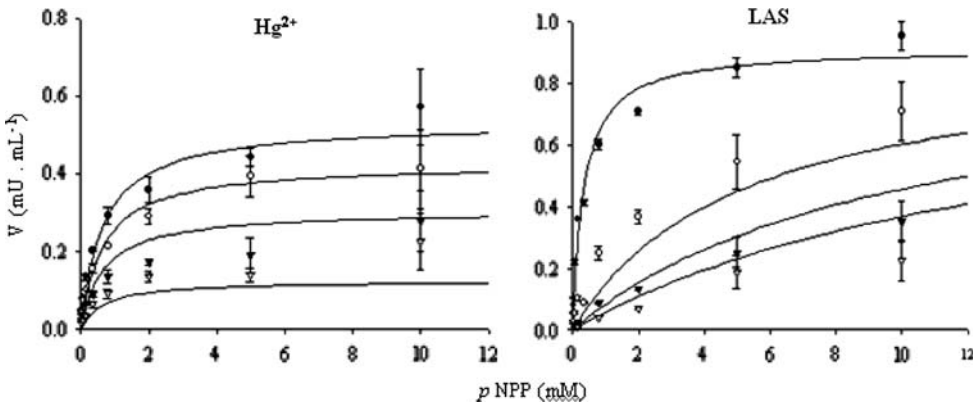

Fig. 3 Effect of Hg^{2+} (● 0.0; ○ 0.01; ▼ 0.03 and ▽ 0.13 mM) and LAS (● 0.0; ○ 0.1; ▼ 0.2 and ▽ 0.3 mM) on *P. subcapitata* acid phosphatase activity at different concentrations of the substrate. Each *point* represent the average value and standard deviations (*vertical bars*) from two analyzes

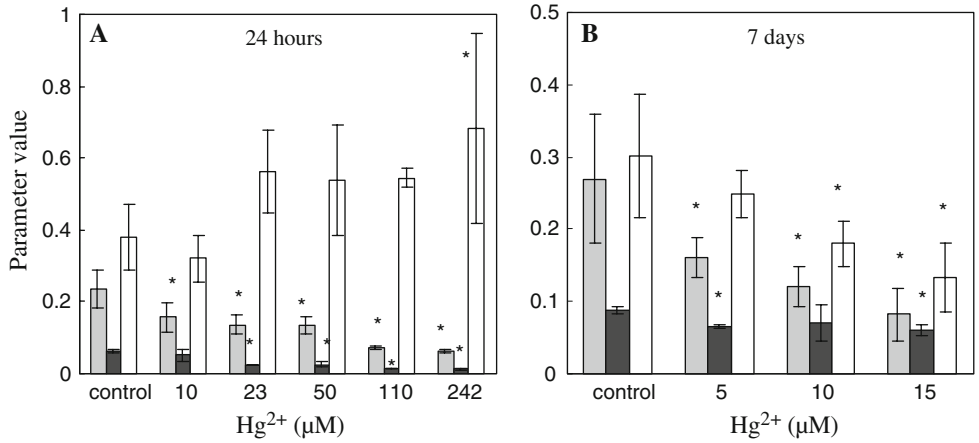

Fig. 4 Biochemical parameters in cultures of *P. subcapitata* exposed to Hg^{2+}. Enzyme activity (▒) (U ml^{-1} × 100), protein concentration (■) (mg ml^{-1}) and specific activity (□) (U mg^{-1} × 10) were determined in extracts from cultures grown in OECD medium after 24 h (**a**) or 7 days (**b**). Average values and standard deviations (*vertical bars*) from two experiments (24 h exposure) and three experiments (7 days exposure) are shown. Analyzes were carried out in duplicate. * Denotes a statistically significant difference ($P < 0.05$) from the control

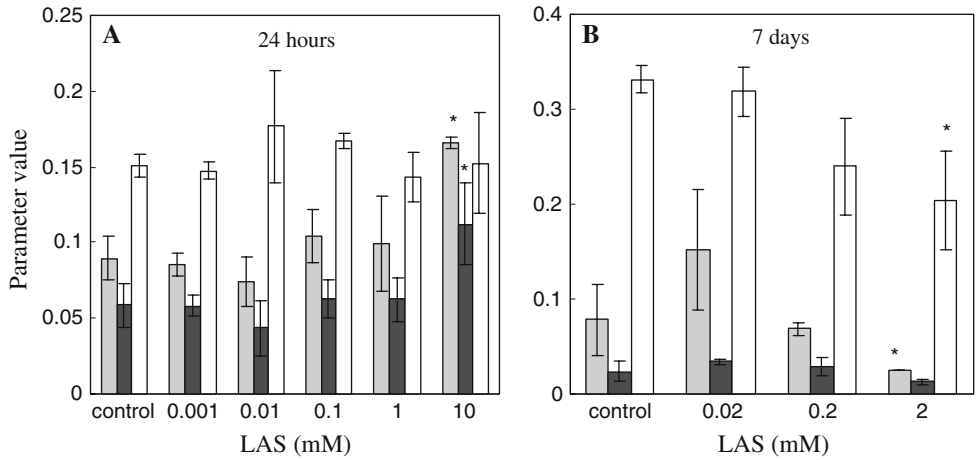

Fig. 5 Biochemical parameters in cultures of *P. subcapitata* exposed to LAS. Enzyme activity (▒) (U ml^{-1} × 100), protein concentration (■) (mg ml^{-1}) and specific activity (□) (U mg^{-1} × 10) were determined in extracts from cultures grown in OECD medium after 24 h (**a**) or 7 days (**b**). Average values and standard deviations (*vertical bars*) from two experiments (24 h exposure) and three experiments (7 days exposure) are shown. Analyzes were carried out in duplicate. * Denotes a statistically significant difference ($P < 0.05$) from the control

were observed in exposed algae cultures. Although both compounds did not change the specific activity in short term exposures, a decrease in activity and protein content was observed in the extracts of Hg^{2+} treatments. On the other hand, these parameters were augmented in LAS exposures for 24 h at the highest concentration and in 7 days exposures at the lowest concentration. These findings are partially consistent with the observed specific activity increase of an alkaline fructose diphosphatase from the Chlorophyceae alga *S. quadricauda* exposed to LAS (Chawla et al. 1987). This observed increment seems to be associated with the tensoactive properties of LAS which could be acting as a total protein extractor adjuvant and aiding in lysosome rupture for acid phosphatase liberation. This is in accordance with Sartory and Grobbelaar (1984) who reported the use of the tensoactive dimethylsulfoxide to extract intracellular substances from green algae. The detergent Triton X-100 has been used to evaluate the lysosomal acid phosphatase latency in algae (Cooper et al. 1974).

According to our short term exposures results, Hg^{2+} affected phosphatase activity and protein content at a median effect concentration 97–235 times lower than that observed for LAS, indicating a strong detrimental effect on these biochemical parameters by the metal.

The increased specific activity observed in Hg^{2+} treatments for 24 h was associated with an increase of the activity/protein ratio. Gill et al. (1991) assumed that this increment could be due to enzyme induction by the metal as part of the biochemical adaptation to meet increased metabolic needs under toxicant induced stress, and/or increased lysosomal liability. Thus, it is well known that enhanced acid phosphatase activity is often associated with increased lysosomal activity in the tissues undergoing cellular degeneration and necrosis due to exposure to toxic substances. Another assumption is that algae seem to be capable of promoting metal-binding to protein as a detoxification mechanism (Omar 2002).

During 7 day experiments, the effects were also more pronounced in Hg^{2+} than LAS exposures. Thus, 2 mM LAS promoted approximately 70% diminution of activity, while the same percentage of effect was attained with 15 μM Hg^{2+}. The inhibitory effect caused by Hg^{2+} was similar in magnitude when compared with that observed for the green alga *Scenedesmus bijuga* acid phosphatase, where about 50% activity was decreased at 2.5 μM Hg^{2+} after 7 days of algae exposure (Fathi 2002).

In contrast to the specific activity depression observed for both pollutants after 7 days exposure, this parameter did not significantly change at 24 h. The calculated EC_{50} value for in vivo Hg^{2+} effect at 7 days exposure was about 7 times lower than the IC_{50} value for in vitro experiments, indicating a major vulnerability of the enzyme in the intracellular medium. A less sensitive effect was observed for the in vivo LAS treatment at 7 days exposure where 67 and 39% decrease of the activity and specific activity, respectively, were attained with 2 mM concentration. The specific activity decrease corroborate with the in vitro inhibition suggesting that the exposure time of cells would be relevant to promote the passage of the chemical across the membranes and the interaction with the enzyme causing its inhibition. This phenomenum was probably hindered at 24 h of exposure where the alterations of enzyme activity in the extract were accompanied by loss of protein content in the Hg^{2+} treatment, or by the enhancement of extractable protein due to the detergent properties of LAS. Thus, the above short term effects on phosphatase activity seem to be the result of other acute toxicant effects rather than the direct intracellular chemical interaction with the enzyme. In other words, the capability of Hg^{2+} and LAS to interact with protein and alter membrane permeability suggest that they acted as general tissue poisons rather than highly specific inhibitors. The same assumption was also supported by Verma et al. (1979) for the toxicity of synthetic detergents.

There is no obvious explanation for the alteration in the specific enzymatic activities after 7 day exposures, since a number of factors may be involved, including depletion in the synthesis of the protein or binding of the enzyme with the chemicals. However, based on the in vitro results, the observed depression in specific activity suggests a pollutant-enzyme interaction.

In comparison to our results, other authors reported the in vivo acid phosphatase activity alteration by metals in unicellular green algae. For example, in *Chlorella vulgaris*, about 20% of activity was depressed by 0.15 mM Al^{3+} (Rai et al. 1998). The specific activity in *S. capricornutum* was decreased about 86 and 70% by 6 μM Al^{3+} and 4.6 μM Zn^{2+}, respectively (Kong and Chen 1995). In *Scenedesmus obliquus*, this inhibition was approximately 40% by 0.12 mM Zn^{2+}, whereas a near 140% specific activity increase was observed in *Scenedesmus quadricauda* exposed to this same condition (Omar 2002).

The diminution of the acid phosphatase specific activity was also reported for exposed fishes, where the pollutant effects were more prominent. Thus, when we compare the inhibition at the highest tested concentration in our work (7 days exposure), a similar degree of effect was attained with 0.44 μM Hg^{2+} in water on the enzyme extracted from gills (Verma et al. 1985) and with 0.014 μM (Misra et al. 1991) or 0.04 mM (Mohan and Verma 1981) LAS for the gill and intestinal acid phosphatase, respectively. This can be explained by the presence of the cell wall in algae which is absent in fish cells. According with Prasad et al. (1998), who studied the tolerance of the wild-type *Chlamydomonas. reinhardtii* cells in comparison with cell wall deficient mutants, wall and synthetized metal-binding complexes

(phytochelatins) might have a defensive function against heavy metal toxicity in algae. The cell wall appears to be serving as a barrier for the cellular membrane and the entry of toxic agents to the cytosol. With regards to the effects of LAS on cell membranes, this barrier may interfere in the change of lipid packing density and in increase fluidity caused by surfactants (Marcelino et al. 2007).

The estimated Hg^{2+} or LAS EC_{50} values for the analyzed biochemical parameters are higher than those reported for the classical growth inhibition test in algae (WHO 1996; 1989). This suggests a higher sensibility of this last physiological parameter in comparison with the enzyme inhibition. The EC_{50}-24 h (9.5 μM) for LAS based on algae glycerol content was also a higher sensitivity parameter (Utsunomiya et al. 1997).

Our results showed that the highest concentration tested of Hg^{2+} and LAS that did not promote a significant effect on specific activity was 5,000 and 143-fold higher, respectively, than the maximum allowed concentration in freshwater compartments, according to Brazilian legislation (CONAMA 2005).

Conclusion

Among the selected pollutants potentially present in sewage sludge, Hg^{2+} and LAS caused the highest degree of in vitro inhibition on the alga *P. subcapitata* acid phosphatase.

Based on the concentrations values of the pollutant agents that promote 50% of enzyme inhibition (IC_{50} and EC_{50} for in vitro and in vivo studies, respectively), Hg^{2+} was more effective than LAS in both conditions.

Despite the similar order of magnitude of IC_{50} values for both pollutants, data suggest higher inhibition by Hg^{2+} than LAS. Thus, the IC_{50} value of the former was minor and confidence limits for each pollutant did not overlap.

We expect that the results of this work can improve our knowledge about the toxicity mechanism and effect of environmental toxicants in relevant biochemical process as occurring in primary producers.

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