Aquatic Toxicology 134–135 (2013) 82–91

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Short-term toxicity of hexavalent-chromium to epipsammic diatoms of a microtidal estuary (Río de la Plata): Responses from the individual cell to the community structure

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ARTICLE INFO

Article history:
Received 4 November 2012
Received in revised form 27 February 2013
Accepted 3 March 2013

Keywords:
Epipsammon
Diatoms
Hexavalent-chromium exposure
Rio de la Plata estuary
Argentina

ABSTRACT

Diatoms are an integral and often dominant component of the benthic microalgal assemblage in estuarine and shallow coastal environments. Different toxic substances discharged into these ecosystems persist in the water, sediments, and biota for long periods. Among these pernicious agents, the toxicity in diatoms by metal is linked to different steps in the transmembrane and internal movements of the toxicant, causing perturbations in the normal structural and functional cellular components. These changes constitute an early, nontaxonomic warning signal that could potentially serve as an indicator of this type of pollution. The aim of this work was to study the environment-reflecting short-term responses at different levels of organization of epipsammic diatoms from the Río de la Plata estuary, Argentina that had been exposed to hexavalent chromium within experimental microcosms. To this end we monitored: (i) changes in the proportion of the diatoms in relation to other algal groups at the biofilm community level; (ii) shifts in species composition at the diatom-assemblage level; (iii) projected changes in the densities of the most representative species at the population level through comparison of relative growth rates and generation times; and (iv) the cytological changes at the cellular and subcellular levels as indicated by the appearance of teratological effects on individuals and nuclear alterations. The epipsammic biofilms were exposed for 96 h to chromium at a concentration similar to that measured in highly impacted sites along the coast (80 $\mu g\,L^{-1}$). Chromium pollution, at this concentration and short exposure time did not affect the algal biomass and density of these mature biofilms. The biofilm composition, however, did change, as reflected in a decline in cyanophytes and an increment in the proportions of diatoms and chlorophytes; with *Hippodonta hungarica*, *Navicula novaesiberica*, *Nitzschia palea*, and *Sellaphora pupula* being the most frequent and abundant species. The most notable shifts related to chromium exposure were a decrease in the relative abundance of *H. hungarica* and a significant increase in the proportion of *N. palea*. Moreover, the species analyzed in the treatment microcosms showed higher growth rates than in the controls – *N. palea* grew faster, while *H. hungarica* replicated more slowly. The total nuclear abnormalities – as recorded in *Fallacia pygmaea* and *N. novaesiberica* – were significantly higher in the treatment microcosms; whereas in *N. palea*, the dominant species in treatment microcosms, neither nuclear alterations nor abnormal frustules were observed.

1. Introduction

The Earth's ecosystems are being transformed under the pressure of large-scale human-induced environmental modifications. Global climate change, eutrophication, discharges of hazardous substances, introductions of alien species, and overexploitation of natural resources affect the health of organisms, community composition, and food-web interactions in aquatic systems with an accelerating rapidity. The earth's largest brackish-water bodies are particularly sensitive to such changes. Different nocuous substances discharged into these ecosystems circulate in the water, sediments, and biota for long periods of time. Live microphytobenthic and phytoplankton communities are species-rich and often dominated by diatoms. These communities are sensitive indicators of environmental change, and small modifications of environmental conditions usually result in measurable species shifts, a feature which is used in environmental-monitoring programs worldwide. Moreover, these microproducers are a source of food for numerous other organisms within the upper trophic levels. Alterations

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http://dx.doi.org/10.1016/j.aquatox.2013.03.007

in these ground-floor microorganisms may therefore disrupt the balance of the whole ecosystem (Snoeijs and Weckström, 2010; Stevenson and Pan, 1999).

Diatom indices reflect the biological status of streams with reference to trophy, acidity, conductivity, and other parameters; but on the whole do not take into account toxic pollution. Field studies dealing with metal contaminations in various regions and countries have shown quite consistent responses of diatom communities (Morin et al., 2012); such as higher abundances of small-sized species (Cattaneo et al., 1998, 2004), increasing proportions of metal-tolerant species, or significantly higher occurrences of valve deformities. Metal toxicity in diatoms is linked to different steps in the circulation of the toxicant both across the membrane (especially with respect to the uptake mechanisms) and inside the cell (e.g., nuclear alterations), thus inducing perturbations in the normal functioning of structural and functional intracellular components. Nevertheless, the literature dealing with the intracellular-component responses to toxic agents is quite limited for freshwater diatoms; organelles are stringently interlinked, and a single alteration in one can seriously perturb the functioning of all the rest (Debenest et al., 2010). These authors pointed out that an understanding of the details of intracellular toxicity in diatoms and the relation between the corresponding intracellular modifications and the disturbance of species composition in the communities represented key topics for further research.

Although the effects of toxic pollution is often not reflected by the structural parameters of taxocenosis or in water-quality indices, the entry of a toxic substance into the cell can produce a series of cytological changes that constitute an early nontaxonomic warning signal with the potential of serving as an indicator of the presence of some type of pollution. The effect of pollutants in aquatic ecosystems can be assessed on several scales (Serra, 2009). Most ecotoxicological tests are performed in the laboratory on small populations of certain species; and although providing useful information on toxicant effects, those assays are not fully reliable in forecasting eventual impacts within natural systems (Cairns and Niederlehner, 1995; Navarro et al., 2002). Tests on single species do not enable an understanding of the effects of toxicants at the community level (Sabater et al., 2007) and furthermore lack ecological realism (Adams et al., 2000; Lagadic et al., 1994; NRCC, 1985). By contrast, tests on natural communities appropriately reflect the ecological reality of a natural system (Cairns and Niederlehner, 1987). A clear definition of the effects of a single metal on diatom communities is, moreover, highly difficult: Several elements are usually present in combination within the environment, and the only way to determine cause-and-effect relationships between the metal and the diatom assemblages is to use artificial microcosms (Falasco et al., 2009).

The controlled environments – such as micro- or mesocosms – offer an opportunity to perform ecosystem-level research in easily replicated test systems under conditions that are manageable in terms of costs and logistics (Roussel et al., 2007).

In such an experimental model the impacts of metal pollution most frequently observed on periphytic algae are addressed at different organizational levels, from the individual cell to the community structure (Morin et al., 2012). Diatoms are an integral and often dominant component of the benthic microalgal assemblage in estuaries and shallow coastal water (Admiraal, 1984). In the Río de la Plata in particular, the diatoms constitute one of the main algal groups in microbenthic, epiphytic and plankton communities (Gómez et al., 2003, 2004, 2009, 2012; Licursi et al., 2010).

The high degree of urbanization and industrialization that is concentrated along the coast of the freshwater tidal zone of the Río de la Plata estuary, and mostly on the Argentine side, generates inputs of contaminants – including nutrients, organic matter, metals (mainly chromium and lead), pesticides, hydrocarbons, suspended solids, and pathogenic agents – that represent a menace both to the biota present and to human health (FREPLATA, 2005).

The aim of this investigation was to study the cause-and-effect relationship between environmental perturbations in the form of hexavalent-chromium inputs and the short-term responses at different levels of organization on the part of epipsammic diatoms from the Río de la Plata within experimental microcosms. To this end we monitored (i) at the biofilm-community level, changes in the proportion of the diatoms in relation to other algal groups; (ii) at the diatom-assemblage level, shifts in species composition; (iii) at the population level, projected changes in the densities of the most representative species through estimations of growth rates and generation times; and (iv) at the cellular and subcellular levels, cytologic alterations as indicated by the appearance of teratological individuals and nuclear abnormalities.

We accordingly exposed these epipsammic biofilms to concentrations of chromium similar to those measured in highly impacted sites along the coast. This metal was selected as the toxic substance to be tested because chromium is commonly found in high concentrations within the coastal areas of the Río de la Plata close to industrial discharges and sewage outfalls (FREPLATA, 2005; INA, 2011).

2. Materials and methods

2.1. Study area

The Rio de la Plata is an extensive, shallow, and microtidal coastal-plain estuary on the southeastern coast of South America. The isohaline region of 0.5 practical salinity units constitutes the boundary between the freshwater and mixohaline zones (Mianzan et al., 2001). The whole study area has a eutrophic condition, and the sediment composition consists mainly in both fine and very fine sand (Gómez et al., 2009).

The Southern Coastal Fringe of the Rio de la Plata is exposed to a variety of contaminants that often have much higher concentrations than those established by the law of conservation of aquatic life (AA-AGOSBA-SHN-ILPLA, 1997). In this zone the highest concentrations of chromium are discharged by the Matanza-Riachuelo (590 kg day^{-1}) and Luján rivers (340 kg day^{-1}) and the Sarandí (270 kg day^{-1}) and Santo Domingo (190 kg day^{-1}) channels. Values of 80–100 μg L^{-1} were measured in water samples from areas close to these discharges (INA, 2011).

2.2. Sample collection and experimental conditions

On December 2010, at a least impacted site with minimal pollution and good biotic integrity (Gómez et al., 2012), epipsammic samples of the intertidal zone were taken at low tide by collecting the surface layer (0.5 cm) with a core (area: 1.5 cm^2). The collected samples were placed in acrylic well-containing microplates and transported to the laboratory in a moist atmosphere.

In the laboratory, the microplates were placed in glass 1.5-L microcosms filled with water collected in the estuary, filtered through net, and finally used for recirculation by means of water pumps (Chosen PH2024 6 W 600 L h^{-1} maximum) to simulate the effect of the tide – i.e., at two cycles per day – (Fig. 1).

The experiment was carried out in a laboratory incubator (SEMEDIC I-500PF) under controlled conditions of temperature (20 °C), light–dark-cycle photoperiod (16L:8D; light intensity of 350 μmol s^{-1} m^{-2}), and immersion–emersion cycles. Tides were simulated as follow: low tide: 4 hs (9 pm–1 am, and 9 am–1 pm); high tide: 8 hs (1 am–9 am, and 1 pm–9 pm).

The experimental design included an acclimation period (3 days), an exposure period (4 days), and a total of 6 microcosms (3 for

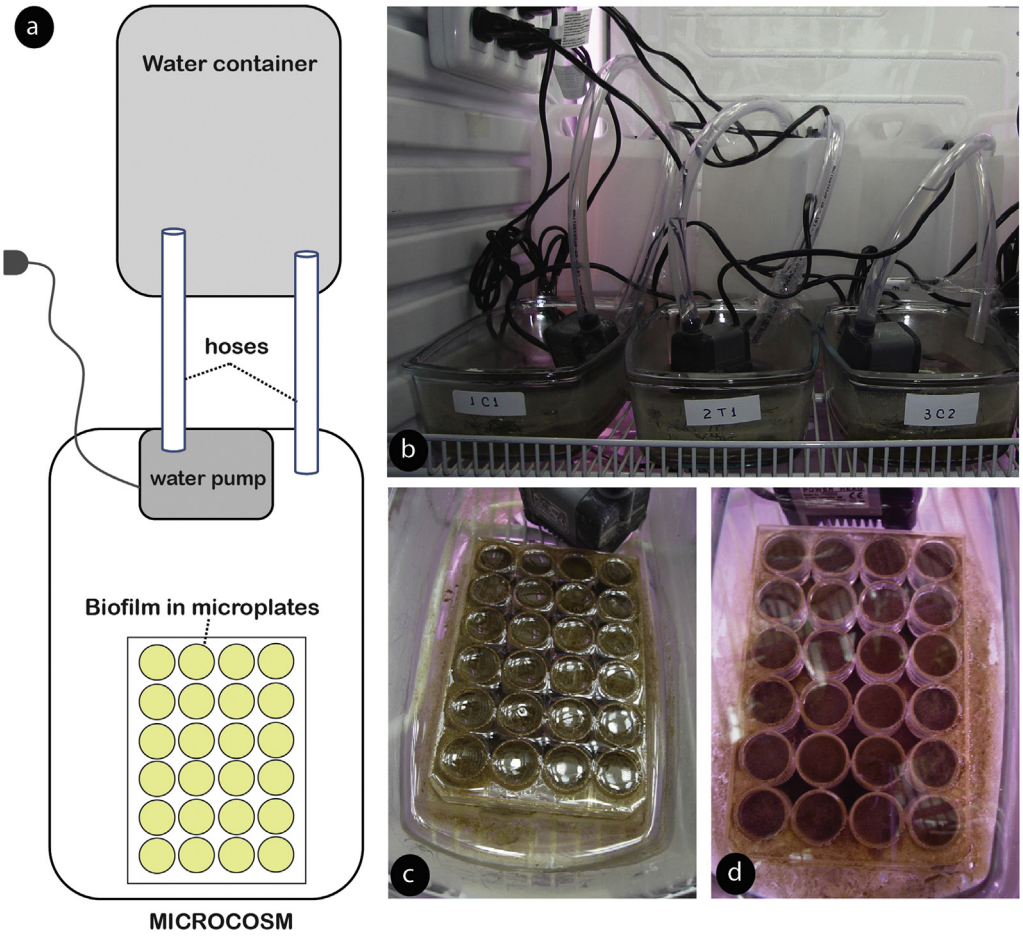

Fig. 1. (a) Design of microcosm. (b) Experimental microcosms placed in the laboratory incubator. Detail of microcosm during (c) low- and (d) high-tide simulation.

control and 3 for treatment conditions). After the acclimation, the water of the microcosms was completely replaced, with hexavalent chromium added to the water of the treatment microcosms at a concentration of 80 $\mu g\,L^{-1}$, corresponding to the chromium concentration reported by FREPLATA (2005) and Lammel et al. (1997) for the most polluted sites of the coast of the Río de la Plata.

Two times during the experiment (at the end of acclimation [t = (day) 3], and at the conclusion of experiment [t = (day) 7]) biofilm samples were collected by pipetting (1.54 cm^2) the sediments from the microplates (randomly selected) for determination of the pigments, the ash-free dry weight (AFDW), and the biofilm composition along with an estimation of the cytological alterations present.

2.3. Physical and chemical characterization

On each day during the experiment, we measured the conductivity (Lutron 4303-CD), dissolved oxygen concentration (ESD 600), temperature, and pH (Hanna HI 8633) in each microcosm.

We removed water samples from the microcosms on days 0, 3, and 7 to determine the nutrient concentrations (soluble reactive phosphorus, nitrate, and ammonia nitrogen) according to Mackereth et al. (1978) and be certain of adequate nutrient availability to the algae throughout the experiment.

2.4. Chromium analysis

The total and hexavalent chromium were determined in the water and the sediment samples collected in the field and in the water and the sediments of the control and the treatment microcosms following Clesceri et al. (1998). The chromium concentrations were analyzed by atomic absorption spectrophotometry according to APHA (1992) and the US Environmental Protection Agency (1986). The detection limits for the water and the sediment were 5 $\mu g\,L^{-1}$ and 0.1 $\mu g\,g^{-1}$ for hexavalent chromium and 2 $\mu g\,L^{-1}$ and 0.5 $\mu g\,g^{-1}$ for total chromium, respectively.

2.5. Chlorophyll a and pheophytins

We collected two sediment samples from each microcosm (experimental unit) at the end of acclimation [t = (day) 3], and at the conclusion of experiment [t = (day) 7] to estimate chlorophyll *a* and pheophytins spectrophotometrically in 90% (v/v) aqueous acetone after Clesceri et al. (1998).

2.6. AFDW

We used two sediment samples collected from each microcosm at the end of acclimation [t = 3], and at the conclusion of experiment [t = 7]) to determine the AFDW according to Clesceri et al. (1998).

2.7. Biofilm assessment and identification of diatom species

For the analysis of the microphytobenthic community two samples were collected, from each microcosm at the end of acclimation [t = 3], and at the conclusion of experiment [t = 7], fixed with formalin (final concentration 4% [v/v]), and quantified in a Sedgwick–Rafter chamber by light microscopy (Olympus BX 51 at

400×). The cell densities of the major algal groups were estimated. The data are expressed as the number of living cells per unit area.

The growth of the diatom community was followed by evaluation of the density of live cells and the density of the most abundant and frequent diatom species of the biofilm analyzed in order to assess the shifts in their growth rates upon chromium exposure.

The growth rates were calculated as

$$K' = \mathrm{Ln}\frac{N_2/N_1}{t_2 - t_1}$$

where N_1 and N_2 = the biomass at an earlier time 1 (t_1) and a later time 2 (t_2), respectively (Levasseur et al., 1993).

The divisions per day and the generation, or population-doubling, time were also calculated as:

$$\text{Divisions per day}: \text{Div. day}^{-1} = \frac{K'}{\mathrm{Ln}2}$$

$$\text{Generation time}: \text{Gen'}t = \frac{1}{\text{Div. day}^{-1}}$$

The subsamples to be used for diatom identifications were first cleaned with H_2O_2, then washed thoroughly with distilled water and mounted on microscope slides with Naphrax®.

Four hundred valves from each sample were identified by microscopy with either interference, phase-contrast, or Nomarski-differential-interference-contrast optics at 1000× magnification. The following keys were used for species identification: Krammer (1992, 2000), Krammer and Lange-Bertalot (1986, 1988, 1991a,b), Lange-Bertalot (2000), and Patrick and Reimer (1966, 1975).

2.8. Assessment of cytological alterations in diatoms

Two sediment samples from each microcosm were collected at the end of acclimation [t = 3 d], and at the conclusion of experiment [t = 7 d] to assess the frequency of anomalies in the frustules (cells with abnormal general shape and/or diatoms with deformed valve-wall ornamentation) and nuclear alterations.

To assess abnormal forms a total of at least 1000 frustules was observed by either interference, phase-contrast, or Nomarski-differential-interference-contrast optics at 1000× magnification to record the frequency of abnormalities.

For observation of the nuclei by microscopy the diatoms were stained with 2% (v/v) Hoechst 33342 (CAS No. 23491-52-3, Sigma Chemical Co.) solution, it constituted at 2 g L^{-1}. Nuclear alterations were counted under 600× magnification with an epifluorescence microscope (Olympus BX50) containing a specific filter for DAPI [4′,6-diamidino-2-phenylindole] (U-MWU2, Ex. filter, BP 330–385; Em. filter, BA 420; dicromatic filter, DM 400). A total of at least 1000 cells for each microcosm were counted to determine the frequency of any one of the following nuclear alterations: abnormal nuclear location, fragmentation of the nucleus into multiple parts, and morphologic changes of the nucleus – i.e., a spreading out of the DNA caused by nuclear-membrane breakage (Debenest et al., 2008). For this evaluation, we first considered the different nuclear locations resulting from normal movements during the cell cycle, as reported by Round et al. (2007) for different diatoms, in order to establish whether or not the positions of the nuclei observed were abnormal.

2.9. Statistical analysis

To reveal the effects of chromium exposure, the physicochemical parameters, the amounts of chlorophyll *a* and pheophytin, the AFDW, the densities of the principal algal groups, the percentage of abnormal frustules, and the nuclear alterations were statistically analyzed with a one-way-variance model (ANOVA) by means of the software PAST version 2.12 (Hammer et al., 2001).

Similarity percentage analyses (SIMPER; Clarke, 1993) based on the relative abundance of diatom species were used to determine the percent contribution of each taxon to the average dissimilarity between the samples from the control and the experimental microcosms. This analysis was based on the Bray–Curtis similarity measurement and was performed by the software PAST version 2.12 (Hammer et al., 2001). SIMPER assumes that fundamental information on the multivariate structure of an abundance matrix is summarized in the Bray–Curtis similarities between samples and that by disaggregating these similarities one most precisely identifies the species responsible for particular aspects of the multivariate description (Clarke, 1993; Clarke and Warwick, 2001).

Spearman's rank-order correlation was used to establish the relationships between the relative abundances of the diatom species and total- and hexavalent-chromium concentrations.

3. Results

3.1. Physicochemical characteristics

The amount of total chromium in the water collected in the field and used for recirculation was 29 μg L^{-1}, while the fraction of hexavalent chromium was below detection limits (i.e., <5 μg L^{-1}); while in the sediment collected the total chromium concentration was 7 μg g^{-1} and of hexavalent chromium 0.5 μg g^{-1}, both lower values than those established by the US Environmental Protection Agency (1996) for its recommended water quality criteria as well as by the Argentine Dangerous Wastes Law No 24051 (1993) for the protection of freshwater life.

Table 1 shows physicochemical characteristic of the water used, and of the water during acclimation (t = 0 d to t = 3 d) and during exposition period (t = 3 d to t = 7 d) for pH, conductivity, temperature, % saturation of dissolved oxygen, and nutrients.

Throughout the experiment the amounts of PO_4^{3-}-P and NO_3^--N showed a slight decrease in both the control and the treatment microcosms, but neither were these decreases significant nor were the amounts at any time growth-limiting (Table 1). Meanwhile during the experiment the amount of NH_4^+-N increased slightly in all the microcosms.

Throughout the experiment the pH, water temperature, and percent dissolved oxygen increased slightly, while the conductivity underwent a slight decrease in both the control and the treatment microcosms (Table 1). ANOVAS revealed no significant differences between the data from the two microenvironments in any of physicochemical parameters measured, nor in acclimation neither in exposition period.

Table 2 shows the average concentrations of total and hexavalent chromium in the water and sediment of the control (C) and treatment (T) microcosms at the end of exposure period.

3.2. Biofilm analysis

The AFDWs were not significantly different between the treatment and the control groups.

The chlorophyll-*a* concentration increased during the experiment in both the control and treatment microcosms, but the rise was significant only in the latter. In contrast, the pheophytins also increased significantly throughout experiment, but in both the control and the treatment microcosms (Table 3).

The total algal density increased in both the control and the treatment microcosms, but was slightly greater in the latter group (Fig. 2). A detailed analysis of biofilm composition revealed that in both the control and the treatment microcosms the density

Table 1
Physicochemical parameters and nutrient amounts measured in water used (collected in the field) and in microcosms during the acclimation and exposition periods (means ± SD).

	pH	Conductivity ($\mu S\,cm^{-1}$)	Temperature (°C)	% DO Saturation	PO_4^{3-}-P ($mg\,L^{-1}$)	NO_3^--N ($mg\,L^{-1}$)	NH_4^+-N ($mg\,L^{-1}$)
Water used	8.8	565.1	20.9	78.8	0.620	1.667	0.020
Acclimation ($t=0$ to $t=3$)							
C1	9.4 (±0.2)	655.3(±43.0)	20.1(±0.6)	88.5(±36.3)	0.533	1.101	0.001
C2	9.5(±0.2)	612.3(±33.0)	20.2(±0.7)	92.2(±32.3)	0.726	0.172	0.001
C3	9.0(±0.1)	589.7(±26.0)	20.0(±0.3)	82.6(±25.5)	0.594	2.290	0.001
T1	9.5(±0.2)	632.3(±27.8)	20.2(±0.7)	86.3(±35.3)	0.565	1.642	0.001
T2	9.4(±0.2)	613.7(±21.7)	19.9(±0.3)	92.0(±31.1)	0.525	1.077	0.001
T3	9.0(±0.1)	586.0(±22.6)	20.0(±0.3)	82.9(±22.9)	0.576	2.052	0.001
Exposition ($t=4$ to $t=7$)							
C1	9.4(±0.5)	649.8(±72.5)	21.7(±0.7)	120.0(±14.4)	0.239	0.648	0.010
C2	9.5(±0.6)	587.0(±25.5)	21.5(±1.0)	127.4(±15.7)	0.597	0.386	0.001
C3	8.9(±0.3)	570.3(±23.7)	20.9(±0.7)	100.9(±11.7)	0.570	1.404	0.001
T1	9.4(±0.5)	582.0(±31.6)	21.6(±0.9)	123.2(±19.2)	0.309	0.433	0.012
T2	9.6(±0.6)	586.0(±26.9)	21.3(±1.2)	126.8(±13.2)	0.251	0.322	0.001
T3	8.8(±0.2)	573.8(±22.3)	20.6(±0.9)	95.2(±8.1)	0.516	0.558	0.009

C1, C2, C3: control microcosms; T1, T2, T3: treatment microcosms. DO: dissolved oxygen.

Table 2
Concentrations of total and hexavalent chromium (means ± SD) in water and sediment of control (C) and treatment (T) microcosms at the end of exposure period.

	Water		Sediment	
	Total chromium ($\mu g\,L^{-1}$)	Cr^{+6} ($\mu g\,L^{-1}$)	Total chromium ($\mu g\,g^{-1}$)	Cr^{+6} ($\mu g\,g^{-1}$)
C	3 (±2)	5(±0.4)	3.8(±0.3)	<D.L.
T	36 (±11)	40(±11)	2.3(±0.5)	<D.L.

D.L.: detection limit ($0.1\,\mu g\,g^{-1}$).

Table 3
Ash-free dry weight (AFDW), chlorophyll-*a* and pheophytin (means ± SD) contents in control (C) and treatment (T) microcosms, before (b) and after (a) chromium addition.

	C b	C a	T b	T a
AFDW ($mg\,cm^{-2}$)	8.0 (±1.6)	5.4 (±1.3)	6.6 (±1.1)	5.6 (±1.3)
Chlorophyll "a" ($\mu g\,cm^{-2}$)	3.1 (±1.5)	3.6 (±1.1)	2.1 (±0.4)	3.8 (±0.8)*
Pheophytins ($\mu g\,cm^{-2}$)	0.6 (±0.3)	1.8 (±0.7)*	0.5 (±0.2)	1.6 (±0.3)**

* Statistical significance $p<0.01$.
** Statistical significance $p<0.001$.

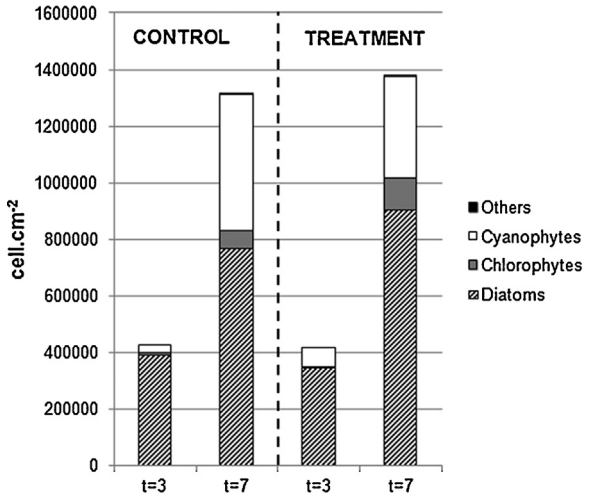

Fig. 2. Densities (expressed as cell cm^{-2}) of major algal groups in the control and treatment microcosms at the end of the acclimation period ($t=3$ d) and at the conclusion of the experiment ($t=7$ d). "Others" is the sum of Euglenophytes, Dynophytes and Charophytes.

of cyanophytes increased throughout the experiment (Table 4). However, in control microcosms cyanophytes made up 36% of the total; whereas in the treatment microcosm the proportion was 26%. Diatoms were the group of algae, which increased most in exposed group (reaching 66% of the total). Also the proportion of chlorophytes increased (up to a final 8%).

The percentage of empty frustules increased significantly ($p<0.01$) throughout the experiment in both the control and the treatment microcosms (reaching 10% at $t=3$ d and 30% at $t=7$ d).

3.2.1. Diatom assemblage

In the microcosm samples 49 diatom species were identified (Table 5). *Hippodonta hungarica*, *Navicula novaesiberica*, *Nitzschia palea*, and *Sellaphora pupula* were the most frequent and abundant species in both the control and the treatment microcosms (Table 5 and Fig. 3). Since, according to the SIMPER analysis, these species reached an 80% cumulative contribution within the comparisons between the control and the treated microcosms (Table 6), the abundances of those four were evaluated throughout the experiment.

These key relative abundances changed in the treatment microcosms as compared to the controls with respect to the proportion of the species within the two groups' assemblages (Table 6). The most

Table 4
Densities (expressed as cell cm^{-2}) of major algal groups in the control (C) and treatment (T) microcosms before (b, [$t=3$]) and after (a, [$t=7$]) chromium addition.

	C b	C a	T b	T a
Diatoms	389,975.1(±63,818.1)	767,920.8(±384,878.8)	342,319.2(±87,278.1)	904,485.4(±319,318.8)
Chlorophytes	9330.4(±8406.8)	64,338.2(±22,637.9)	7648.7(±3697.8)	112,979.9(±86,519.5)
Cyanophytes	28,495.3(±23,523.6)	478,259.5(±191,783.9)	66,287.9(±72,989.2)	357,182.6(±183,363.8)
Euglenophytes	0(±0)	99.5(±243.7)	0(±0)	89.5(±219.3)
Dynophytes	0(±0)	0(±0)	0(±0)	238.7(±584.8)
Charophytes	0(±0)	0(±0)	0(±0)	1293.1(±3167.5)
n	6	6	6	6

Table 5
List of diatoms identified in the control and treatment microcosms. Species are listed in order of decreasing frequency.

Species	Acronym	Freq
Hippodonta hungarica (Grunow) Lange-Bertalot Metzeltin & Witkowski	HHUN	***
Navicula novaesiberica Lange-Bertalot	NNOV	***
Nitzschia palea (Kützing) W.Smith	NPAL	***
Sellaphora pupula (Kützing) Mereschkowksy	SPUP	***
Craticula halophila (Grunow ex Van Heurck) Mann	CHAL	***
Hippodonta capitata (Ehrenberg) Lange-Bert.Metzeltin & Witkowski	HCAP	***
Fallacia pygmaea (Kützing) Stickle & Mann ssp. *pygmaea* Lange-Bertalot	FPYG	***
Navicula sanctaecrucis Ostrup	NSTC	***
Neidium ampliatum (Ehrenberg) Krammer	NEAM	**
Stauroneis brasiliensis (Zimmerman) Compere	STBR	**
Navicula erifuga Lange-Bertalot	NERI	**
Nitzschia levidensis (W. Smith) Grunow in Van Heurck	NLEV	**
Navicula monoculata Hustedt var. *omissa* (Hustedt) Lange-Bertalot	NMOM	**
Navicula gastrum (Ehrenberg) Kützing	NGAS	**
Hantzschia virgata (Roper) Grunow var. *virgata*	HVIR	**
Nitzschia lacunarum Hustedt	NLCR	**
Navicula constans Hustedt var. *symmetrica* Hustedt	NCSY	**
Amphora libyca Ehrenberg	ALIB	**
Navicula viridula var. *germainii* (Wallace) Lange-Bertalot	NVGE	**
Navicula gregaria Donkin	NGRE	*
Navicula humboldtiana Lange-Bertalot & Rumrich	NHUB	*
Nitzschia desertorum Hustedt	NDES	*
Navicula veneta Kützing	NVEN	*
Navicula monoculata Hustedt	NMOC	*
Craticula pampaeana (Frenguelli) Lange-Bertalot	CRPA	*
Nitzschia umbonata (Ehrenberg) Lange-Bertalot	NUMB	*
Fallacia clepsidroides Witkowski	FCLE	*
Sellaphora nyassensis (O.Muller) D.G. Mann	SNYA	*
Placoneis placentula (Ehrenberg) Heinzerling	PPLC	*
Nitzschia frustulum (Kützing) Grunow var. *frustulum*	NIFR	*
Navicula viridula (Kützing) Ehrenberg var. *rostellata* (Kützing) Cleve	NVRO	*
Navicula recens (Lange-Bertalot) Lange-Bertalot	NRCS	*
Navicula tenelloides Hustedt	NTEN	*
Nitzschia capitellata Hustedt	NCPL	*
Geissleria schmidiae Lange-Bertalot & Rumrich	GSHM	*
Placoneis clementis (Grunow) Cox	PCLT	*
Craticula accomoda (Hustedt) Mann	CRAC	*
Navicula citrus Krasske	NCIT	*
Nitzschia inconspicua Grunow	NINC	*
Navicula arvensis Hustedt	NARV	*
Nitzschia gracilis Hantzsch	NIGR	*
Eolimna subminuscula (Manguin) Moser Lange-Bertalot & Metzeltin	ESBM	*
Achnanthes lanceolata ssp. *rostrata* (Oestrup) Lange-Bertalot	ALAR	*
Caloneis bacillum (Grunow) Cleve	CBAC	*
Achnanthes lanceolata (Brebisson)Grunow var. *lanceolata* Grunow	ALAN	*
Amphora montana Krasske	AMMO	*
Navicula molestiformis Hustedt	NMLF	*
Pinnularia gibba Ehrenberg	PGIB	*
Gomphonema parvulum (Kützing) Kützing var. *parvulum*	GPAR	*

* Freq: % of frequency in the total sample dataset: <10.
** Freq: % of frequency in the total sample dataset: 10–20.
*** Freq: % of frequency in the total sample dataset: >20.

noticeable shifts related to metal exposure were a decrease in the relative abundance of *H. hungarica* and an increase in the proportion of *N. palea*. In addition, the relative abundance of *N. palea* was significantly correlated ($R = 0.68$, $p < 0.05$) with the concentration of hexavalent chromium. *N. novaesiberica* and *S. pupula* exhibited no notable changes between control and treated mesocosms.

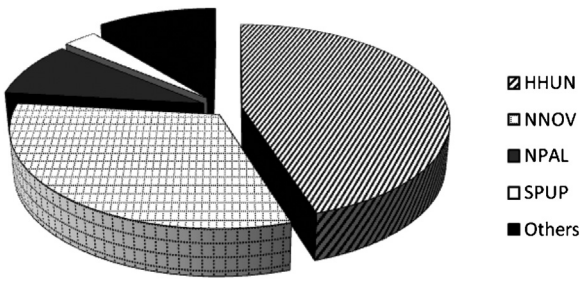

Fig. 3. Average relative abundance of diatom assemblages at the end of the acclimation period ($t = 3$ d). Species acronyms are given in Table 5.

3.2.2. Growth rates

Although no significant differences in the growth rates were observed, the species analyzed did grow somewhat faster in the treatment microcosm than in the control microenvironment (Table 7). *N. palea* showed the highest growth rate (control:

Table 6
Average abundance of species contributing at high percentages to the dissimilarity (27.70%) between the control and treatment microcosms at the end of the experiment ($t = 7$). The species acronyms are given in Table 5.

Taxon	Control	Treatment	Contribution	Cumulative %
HHUN	45.40	25.10	10.16	36.73
NPAL	17.40	34.80	8.97	69.16
NNOV	25.30	28.60	2.78	79.19
SPUP	3.11	3.86	1.24	83.66

Table 7
Growth rate. divisions per day and generation time for *Navicula novaesiberica* (NNOV). *Hippodonta hungarica* (HHUN). *Nitzschia palea* (NPAL) and *Sellaphora pupula* (SPUP) in control (C) and treatment (T) microcosms. SD in brackets.

	Growth rates		ANOVA			Divisions per day		Generation time	
	C	T	*F*	*p*	*n*	C	T	C	T
NNOV	0.18(±0.12)	0.23(±0.05)	0.76	0.41	12	0.26(±0.18)	0.33(±0.07)	3.88	3.02
HHUN	0.05(±0.09)	0.12(±0.11)	1.42	0.26	12	0.07(±0.13)	0.17(±0.15)	15.11	6.02
NPAL	0.33(±0.25)	0.45(±0.15)	0.83	0.38	12	0.48(±0.36)	0.65(±0.22)	2.08	1.54
SPUP	0.14(±0.14)	0.22(±0.13)	1.01	0.34	12	0.20(±0.21)	0.32(±0.18)	4.91	3.09

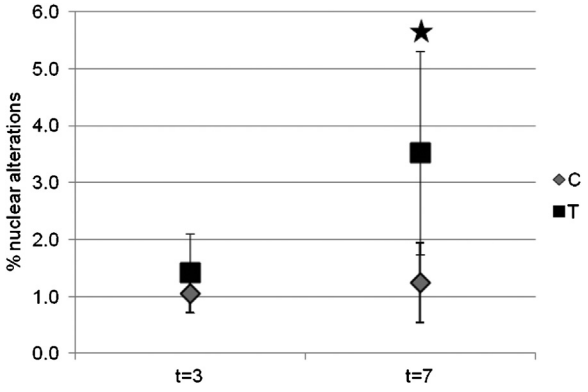

Fig. 4. Percentage of nuclear alterations (sum of abnormal nuclear location, nuclear membrane breakage, and nuclear fragmentation) of diatoms from the control and treatment microcosms at the end of the acclimation period ($t = 3$ d) and at the conclusion of the experiment ($t = 7$ d). statistical difference from the control: $p < 0.01$.

0.33 divisions d^{-1} vs. treatment: 0.45 divisions d^{-1}) with the lowest values being recorded for *H. hungarica* (control: 0.04 divisions d^{-1} vs. treatment: 0.11 divisions d^{-1}). The number of divisions per day and the generation times measured for these species were consistent with the faster replication of the algae in the treatment than in the control microcosms (Table 7).

3.2.3. Cytologic anomalies

The percentage of nuclei with abnormal locations underwent a significant increase ($p < 0.05$) in the diatom assemblages exposed to hexavalent chromium (Fig. 4). A significant increase ($p < 0.05$) in the percentage of cells with nuclear membrane breakage was also observed in the treatment microcosms upon chromium exposure, and this value became significantly higher ($p < 0.01$) in the treatment than in control microcosms at the conclusion of the exposure ($t = 7$; Fig. 5). Abnormal nucleus locations and nuclear membrane breakage were recorded only in specimens of *Fallacia pygmaea* and *N. novaesiberica* (Fig. 6); no such anomalies were recorded in other species observed. The incidence of nuclear fragmentation, however, was not significantly higher in the treatment microcosms than in the controls. Abnormal frustules were not observed in either microcosm.

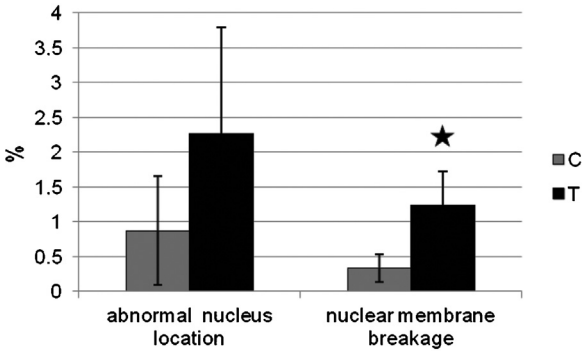

Fig. 5. Percentage of abnormal nuclear location and nuclear membrane breakage of diatoms from control and treatment microcosms at the conclusion of the experiment ($t = 7$d). statistical difference from the control: $p < 0.01$.

4. Discussion

The microphytobenthic community analyzed in this study exhibited an increase in chlorophyll-*a* levels and algal densities in both the control and the treatment microcosms without significant differences between the two microenvironments. Exposure to chromium – at the concentration tested and during a short-term period (i.e., 96 h) – would therefore appear not to have affected the algal biomass or the density of the mature biofilms (those having

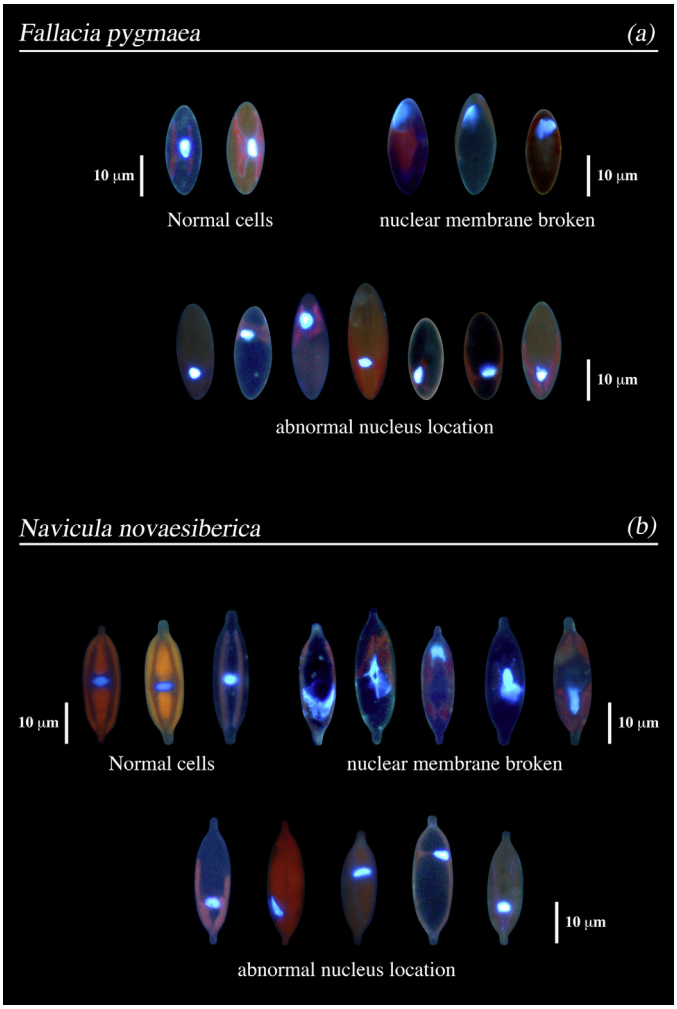

Fig. 6. Cells of *Fallacia pymaea* (a) and *Navicula novaesiberica* (b) with normal and abnormal nuclear locations and with broken nuclear membranes observed under the epifluorescence microscope (nucleus stained in blue with Hoechst 33342, chloroplasts appearing in red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

various growth forms: adnates along with stalked and motile species) in this experiment. In contrast, different authors have reported a decrease in algal biomass related to longer exposures to copper, zinc, and/or cadmium – i.e., from 24 days to 2–12 weeks (Atazadeh et al., 2009; Ivorra, 2000; Morin et al., 2012; Paulsson et al., 2000; Serra and Guasch, 2009; Soldo and Behra, 2000). In agreement with the results here, Duong et al. (2010) reported that a mature biofilm's development was not inhibited after two weeks of cadmium contamination and seemed to be more resistant to cadmium toxicity, unlike the significant decrease in biomass they observed in younger biofilms. This difference could be related to the protective function of the biofilm imparted by the three-dimensional architectural design of the diatom assemblages that constitutes a fundamental and integral feature of the biofilm. Consistent with this notion, biofilms in an early colonization stage have been cited as being more vulnerable to metal exposure than mature ones (Ivorra et al., 2000).

That a biofilm's maturity and/or thickness may reduce susceptibility to toxicity is a general rule that has been demonstrated for metals (e.g., Duong et al., 2010) and herbicides (e.g., Franz et al., 2008), as was pointed out by Guasch et al. (2012).

According to the theories of adaptation to stress by algal communities (Stevenson, 1997), the biomass should be less sensitive to environmental stress than the species composition, because communities can compensate for stress by changing composition (Stevenson and Pan, 1999). In our study, the biofilm composition did change as a consequence of chromium exposure, as reflected in the decline in cyanophytes along with an increment in the proportions of diatoms and chlorophytes in the treatment microcosms. The tendency observed during this short-term exposure agrees with the observations of Soldo and Behra (2000), who reported that long-term exposure to copper changed the taxonomic composition of the periphyton communities and that major compositional changes included a decline of the Cyanophyceae and an increase in the Chlorophyta. In particular, the analysis of the diatom assemblage in this experiment indicated that during the acclimation period *H. hungarica*, *N. novaesiberica*, *N. palea*, and *S. pupula* were the most frequent and abundant species in both microcosms. These species have been reported by Licursi et al. (2010) as eutrophic and tolerant to organic matter. The most notable shifts upon chromium exposure were a decrease in the relative abundance of *H. hungarica* and a significant increase in the proportion of *N. palea* in the treatment microcosms. Changes in species composition are frequently reported in studies dealing with metal pollution (Admiraal et al., 1999; De Jonge et al., 2008; Gómez and Licursi, 2003; Guasch et al., 2009; Hill et al., 2000; Hirst et al., 2002; Ivorra, 2000; Sabater, 2000; Takamura et al., 1990). Changes occurring in the species composition of periphyton communities experiencing metal contamination have been thought to result from a selection for those species that are tolerant to that pollution (Niederlehner and Cairns, 1992).

In the present work, the algae monitored in the treatment microcosms had higher growth rates than those in the control microenvironments. Among these species, *N. palea* had the highest values, while *H. hungarica* had the lowest. Several studies have reported lower growth rates in biofilms from metal-polluted sites. As reviewed by Morin et al. (2012), under metal exposure diatom growth can be delayed, or even inhibited, leading to a reduction in the diatom biomass (Gold et al., 2003; Guanzon et al., 1994; Payne and Price, 1999; Pérès, 1996; Perrein-Ettajani et al., 1999).

The total nuclear abnormalities observed in our study were significantly higher in the treatment microcosms; but whereas these aberrations were recorded in the specimens of *F. pygmaea* and *N. novaesiberica*, no such nuclear alterations were observed in *N. palea* – the dominant species in the treatment microcosms. A few studies have been conducted to determine the toxic effects of chemicals on the diatom cellular nucleus (Debenest et al., 2010). Most of those reports cited DNA dispersion in diatom cells; multinuclear cells; the presence of a micronucleus; or DNA fragmentation as a consequence of exposures to aldehydes, herbicides, colchicine, or ultraviolet radiation (Buma et al., 1995, 1996; Casotti et al., 2005; Coombs et al., 1968; Debenest et al., 2008; Duke and Reimann, 1977; Rijstenbil, 2001). To the best of our knowledge, though, the report of Desai et al. (2006) is the only literature precedent assessing the genotoxic effects of metals in diatoms: by comet assays on *Chaetoceros tenuissimus* those authors found that higher cadmium levels produced an early damage to the nuclear material of the cell and that the percentage of that injury increased with progressive exposures.

The absence of nuclear alterations in *N. palea* – along with the higher growth rate of that species as well – emphasizes the high degree of injury resistance and stress tolerance of that diatom in epipsammic biofilms, it being able to survive and reproduce notably during exposure to hexachromium. This resistance of *N. palea* has been reported by several authors (Lai et al., 2003; Medley and Clements, 1998; Morin et al., 2008; Pérès et al., 1997; Whitton, 2003). Furthermore, diatom communities in younger (i.e., immature) biofilms exposed to cadmium were seen to increase their tolerance to cadmium by a highly significant increase in the proportion of *N. palea* (Duong et al., 2010). Diatom species composition is driven by several environmental factors. Among the chemical parameters, the exposure to toxic agents such as metals can be a major determinant; but because the impacts caused specifically by metals are generally difficult to separate from other environmental stressors, no agreement has so far been achieved on the sensitivity or tolerance for any particular given diatom species (Morin et al., 2012). Accordingly, the manner in which diatoms respond to toxic stress, and the magnitude of their response, also depends on cell and community health, on ecological interactions with other organisms, and on general environmental conditions. The fundamental structural parameters of diatom communities (e.g., biomass, cell density) are less sensitive, for example, to pesticide effects than are the specific structural parameters of those unicellular organisms themselves – i.e., the species composition and the density of each species (Debenest et al., 2010).

Metal pollution is one of the main causes of teratological forms in diatoms (Falasco et al., 2009). In the present study abnormal frustules were not observed in either the control or the treatment microcosms. This absence could have resulted from the short-term exposure to hexachromium and from the biofilm's degree of maturity. Duong et al. (2008) reported a frequency of 8‰ abnormal diatom frustules in early-stage biofilms after four days of cadmium exposure; but these abnormalities appeared at a significant level (13‰) only after two weeks of cadmium exposure in mature biofilms (Duong et al., 2010).

The use of microcosms – or mesocosms and even artificial streams – where biofilm communities can develop may achieve the necessary compromise between the simplification of the laboratory setting and the authenticity of the natural system along with the absolutely required reproducibility of the former (Sabater et al., 2007). Modern research into bioindication and biomonitoring should be directed at insuring a greater comparability between the causes and effects produced in the laboratory and the corresponding phenomena occurring in the field by narrowing the differences in threshold concentrations, sensitivities, and extents of reaction (Markert et al., 2003) along with the dissimilarities in the environmental conditions. Consequently, in our study natural biofilms – and as such containing nature's genetic diversity – were exposed to chromium concentrations similar to those found in the polluted sites on the coast of the Rio de la Plata in order to simulate the estuary's natural conditions and thus allow an approximation of the responses observed in laboratory to those that would occur under comparable conditions in nature. Finally, nuclear

abnormalities, changes in biofilm composition and population increase of most tolerant diatoms (i.e., *N. palea*) reveals the effects of hexavalent chromium over the microproducers of the basal trophic levels of this estuary.

Acknowledgements

Financial support for this study was provided by grants CONICET-PIP 2011–2013 Res. 266/10. The authors wish to thank Dr. Donald F. Haggerty, a retired career investigator and native English speaker, for editing the final version of the manuscript, and to Jorge Donadelli for his work with the chemical analyses. Finally, we would like to express our thanks to the anonymous reviewers for improvements in this manuscript. This is ILPLA Scientific Contribution No. 929.

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