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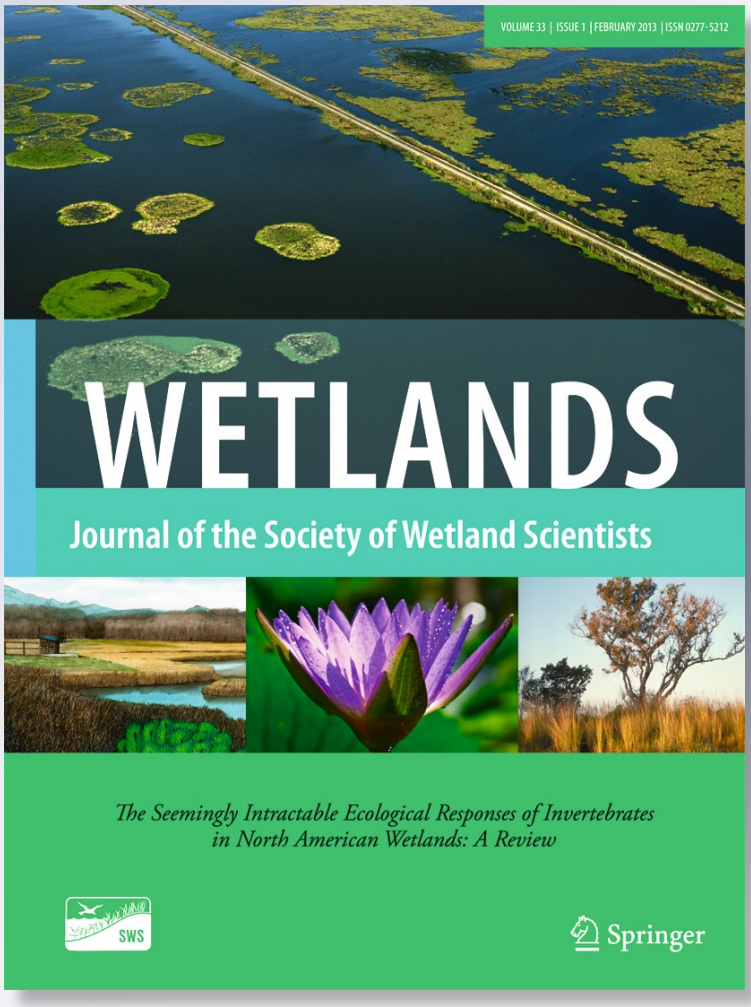

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ARTICLE

Experimental Evidence of the Tolerance to Chlorate of the Aquatic Macrophyte *Egeria densa* in a Ramsar Wetland in Southern Chile

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Abstract In 2004, a massive die-off of the aquatic macrophyte *Egeria densa* occurred in a Ramsar wetland in southern Chile. In 2004, a pulp mill started its operations upstream from the wetland. Chlorate, a chemical compound found in pulp mill effluent, was one of the suspected causes of the observed ecosystem-level changes. The hypothesis was that high concentrations of chlorate in the wetland caused a massive die-off of the large populations of *Egeria densa* in this ecosystem. In this study several experimental efforts were aimed to understanding the potential effect of chlorate on *E. densa*. Plants were exposed to different doses of chlorate for variable periods in a large mesocosm array and several morphometric endpoints were quantified. Additionally, an ecotoxicological assay was implemented providing the first insight into the tolerance to chlorate of this aquatic plant. Both mesocosm and ecotoxicological evidences suggest that *E. densa* is tolerant to fairly high levels of chlorate (i.e. EC50 in the order of 1000 mg/L), at least three orders of magnitude larger than the highest concentration recorded in the wetland. Our results provide evidence that should guide the efforts of understanding the real causes of this environmental change.

Keywords Pulp mill effluent · Chlorate · Water quality · Aquatic macrophyte · Wetland

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Introduction

In 2004, important ecosystem-level changes were detected in the Carlos Anwandter Nature Sanctuary, a large wetland system in southern Chile (40°S) designated as a Ramsar site in 1981.[1] One of the most conspicuous changes affecting the Sanctuary during this period included a massive die-off of the aquatic macrophyte *Egeria densa*, a species originally from southern Brazil (Boettcher 2007) that serves as a major food source to several charismatic species like the black necked swan *Cygnus melancoryphus* and other bird species (Corti and Schlatter 2002). The submerged macrophyte community continued its decline until it had virtually disappeared by October of 2005 (Marín et al. 2009). These ecosystem-level changes occurred by the time a newly installed pulp mill (Valdivia plant) owned by Celulosa Arauco y Constitución S.A. (hereafter referred to as CELCO) started its operations, some 30 km upstream from the wetland. It is still under debate what caused the demise of *Egeria densa* from the system.

The Río Cruces wetland is a nationally and internationally important migratory bird habitat. Besides being the field laboratory for innumerable descriptive Masters and Doctoral theses, the Sanctuary has been monitored and protected by wildlife wardens of the Corporación Nacional Forestal (CONAF) since 1982. Nevertheless, few studies provide reliable information about the wetland's dynamics and the forces that shape its plant and animal communities. Due to this lack, the causes of the sudden biotic changes that occurred in 2004 are unknown. Since then, several scientists analyzed the situation and produced a plethora of possible explanations from different perspectives. While some argumental lines-mainly representing inferences from temporally limited field observation-pointed to chemical changes attributed to the operations of CELCO (i.e. Mulsow and

[1] http://www.ramsar.org.

Grandjean 2006; Lopetegui et al. 2007) or to the effect of physical and biological factors (Pinochet et al. 2004; Ramírez et al. 2006; Woelfl et al. 2006; Lagos et al. 2008), others either disproved the former by emphasizing the lack of appropriate data analyses (Harding et al. 2007; Soto-Gamboa et al. 2007) or provided experimental evidence that refuted some of their arguments (Palma et al. 2008; Marín et al. 2009). Hence, the whole issue has become very controversial.

Various hypotheses were proposed that attributed this environmental change to either natural or man-made causes (UACH 2005; Marín et al. 2009). One of these hypotheses stated that chlorate present in the effluent from CELCO had a toxic effect on *E. densa*, which susequently disappeared, thus triggering the aforementioned environmental change in the wetland. This was suggested after a concentration spike of chlorate was detected in the facility's discharge effluents (values endorsed by governmental authorities) resulting in a maximum estimated concentration of 7.8 mg/L in the Cruces River just downstream of the effluent discharge (see Table 1) during the period ranging from February to April 2004. At the same time, above natural concentrations of chlorate (with a maximum of 0.52 mg/L) were recorded in April of 2004 in the water column at the head of the wetland, located 30 km downstream of CELCO's effluent discharge. This situation is comparable, to some extent, to what occurred in Sweden with the recorded negative effect of chlorate, originated from a pulp mill plant, on a marine brown alga (Rosemarin et al. 1994). In our system, however, the main species negatively affected (presumably by chlorate) was a freshwater macrophyte.

Table 1 Chlorate concentrations on CELCO's effluent during the first months of 2004[a] and the estimation of Chlorate concentrations in the wetland located 32 km downstream (in bold: highest weekly chlorate concentrations recorded)

Week	Chlorate in CELCO's effluent (mg/L)	Estimated chlorate in Cruces River immediately after discharge (mg/L)[b]
Feb 9-Feb 15	17	0.5
Feb 16-Feb 22	24	0.6
Feb 23-Feb 29	28	1.3
Mar 1-Mar 7	19	0.6
Mar 8-Mar 14	**37**	**1.6**
Mar 15-Mar 21	**170**	**7.8**
Mar 22-Mar 28	**90**	**4.6**
Mar 29-Apr 4	**23.7**	**0.9**
Apr 5-Apr 11	CELCO did not operate	–
Apr 12-Apr 18	40	0.4
Apr 19-Apr 25	10.4	0.1
Apr 26-May 2	0.65	0.0

[a] Effluent water quality monitoring report for the Feb-Apr 2004 period. The chemical analyses were performed in CELCO's laboratory at the Valdivia Plant as autocontrols. The analytical procedure involved molecular absorption spectrophotometry (O-toluidine method)

[b] Based on a chlorate mass balance calculation for the Cruces River, involving the point of effluent discharge

Several environmental sources for chlorate are known. Chlorate may leach from agricultural lands where compounds containing chlorate are used as herbicides or defoliating agents (Logan 1998). Chlorate is also a by-product of water disinfection, mainly with chlorine dioxide (USEPA 1999). Compounds containing chlorate are also used by cellulose plants in the production of chlorine dioxide, a common bleaching agent (Logan 1998). In turn, use of chlorine dioxide produces residual concentrations of chlorate ion in the effluents of cellulose plants.

Prior to our study, the only significant toxicity on large primary producers, attributed to chlorate, was demonstrated for brown algae at low levels of this compound (van Wijk and Hutchinson 1995) based on long-term mesocosm studies. In order to protect the most sensitive freshwater species, presumably green algae, the Government of British Columbia, Canada, stated an environmental standard for chlorate of 30 mg/L (Warrington 2002), although long-term toxicity data for freshwater systems is scarce. Comparatively, to protect brown algae, this standard is 0.005 mg/L of chlorate for marine aquatic life, based on long-term toxicity data. Since chlorate and nitrate are structural analogs, the toxicity of chlorate is associated with its reduction to toxic chlorite by the enzyme nitrate reductase (Aberg 1947; Balch 1987; van Wijk and Hutchinson 1995; Stauber 1998; Chaudhuri et al. 2002). Chlorate is regarded as a chemically stable species and mobile in aquatic ecosystems, given its high solubility and poor sorption properties (Siddiqui 1996; Couture 1998; Logan 1998; van Wijk and Hutchinson 1995; USEPA 1999). Chlorate is not persistent though in these systems, since specialized populations of facultative bacteria are known to rapidly reduce chlorate to chloride in anaerobic sediments (Malmqvist et al. 1991; van Ginkel et al. 1995; Logan 1998; Coates et al. 1999; Schwarz et al. 2012). The above mentioned lines of evidence regarding the effect of chlorate on different biological endpoints are diverse and preclude us from disregarding or accepting the hypothesis about the direct negative effect of chlorate on *E. densa*, in particular, in view of the lack of long-term toxicity data for freshwater species. Neither Chile, USA or Canada have emissions or water quality standards for chlorate, only one recommendation from the Canadian environmental authority regarding a limit of chlorate (30 mg/L) for freshwater biota (Warrington 2002). Hence, considering all the available information on ecotoxicity associated to chlorate, no acute toxic effect of chlorate should be expected on *Egeria densa* under the circumstances witnessed in the system in 2004. Hence, the main objective of this research was to test the hypothesis of the lethal and sublethal effect of chlorate on *Egeria densa* under different experimental conditions within

a mesocosm system, which is an amenable tool to answer such questions (i.e. Liston et al. 2008; Palma et al. 2008). The chlorate levels and other abiotic conditions considered in the experiment were selected to recreate several different scenarios likely to have affected this species in its natural environment during March and April of 2004 in the Ramsar wetland. Our general working hypothesis is that plants exposed to chlorate levels equivalent to those present in the natural system by the time the environmental changes in the wetland were evident (early 2004) experience significant negative effects on morphological (e.g., changes in length, biomass, number of roots) as well as physiologically related (i.e. chlorophyll-*a*) endpoints.

A second objective aims to expand the findings of the mesocosm experiment through the use of a more general set of chlorate concentrations in order to understand the toxicity threshold of this compound on *E. densa*.

Methods

A series of experiments, using a mesocosm experimental array, were implemented between 2009 and 2011. In each opportunity, several different morphometric and physiologically related variables were quantified in *E. densa* plants exposed to different concentrations of chlorate. Additionally, we implemented an ecotoxicological assay in order to determine the 50 % effect concentration (EC50) toxicity level of chlorate on *E. densa*.

Description of the Basic Mesocosm System and Experiments

The layout consisted of fifteen 1,000-L, square, polyethylene tanks. Through an interconnected manifold system, each tank received a continuous, gravity-fed flow (≈2 L/min) of water from a 2,000-L head tank filled with water pumped from an uptake situated approximately 100 m upstream from the plant's general effluent discharge. Water drained continuously through a bottom outflow, covered with a 500-μm plastic mesh that was connected to an upward-curved pipe for the control of water volume within the experimental tanks, which for the present study was set to 600 L. Two different mesocosm experiments were run in parallel, with constant level of chlorate during 3 months (chronic exposure) and a slug of chlorate during 1 month (acute exposure). Inside each tank we placed five *E. densa* plants that were kept suspended separately at mid water level, much like plants are sometimes found floating in the water column in its natural environment. The system was divided in three groups of five randomly chosen tanks. Two of these groups received each chronic and acute doses of chlorate (as justified below). Precise dosage of chlorate to each tank was possible by interposing two dispensers (Dosatron™ Clearwater, Florida, USA) at the beginning of each of the pipes feeding the tanks under chlorate treatment. These dispensers were fed with a concentrate solution of chlorate from a 100-L tank which was prepared from sodium chlorate similar to that used by CELCO (Fig. 1). Even though this is a very reliable system that ensures the desired

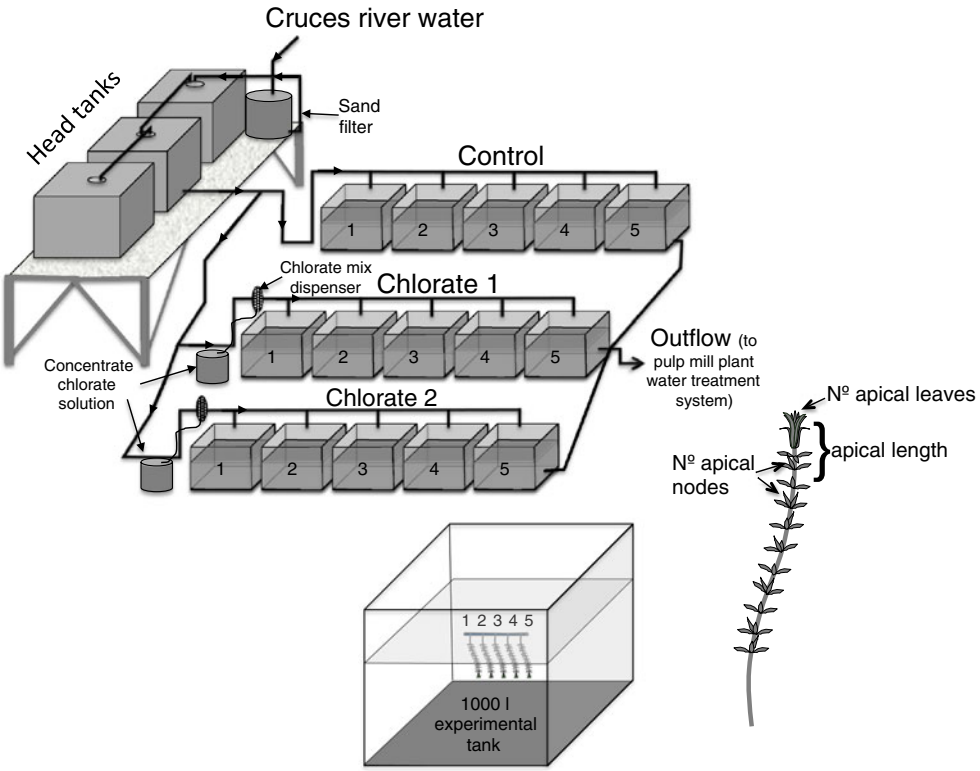

Fig. 1 Mesocosm experimental setup highlighting its main components. In detail is a close-up of 5 plants inside each tank and the type of morphometric measures obtained from each plant

concentration before reaching the experimental tanks, weekly water samples were obtained for chlorate concentration determinations from treatment as well as from control tanks using ion chromatography. Every 3–4 days several physical and chemical variables were measured inside the tanks of both chronic and acute experiments (Table 2).

The experimental design for the mesocosms allowed us to analyze the information from a series of dependent variables (i.e. morphometric variables in plants of *E. densa*) through a repeated measures analysis of variance following a block design (i.e. von Ende 1993; Palma et al. 2008). On a weekly or biweekly basis, for the acute and chronic treatments, respectively, all the plants were individually measured (wet biomass, total length, apical length, number of apical nodes, number of lateral ramifications, number of roots and number of apical leaves). At the end of each experimental trial, chlorophyll-*a* content was determined from apical leaves (100 mg) from a sub-set of plants corresponding to each treatment. Plant tissue was manually macerated with 25 ml of 90 % acetone until a homogeneous green solution was obtained. The solution was then passed through a Whatman GF/F 47 mm diameter glass fiber filter. The content of chlorophyll of this solution was obtained by measuring the absorbance using a spectrophotometer (Hach DR 2700) following the procedure for acetone-extracted samples (Dere et al. 1998) and utilizing the analysis provided by Lichtentaler and Wellburn (1985).

Justification and Criteria Considered for the Chlorate Concentrations Utilized in the Mesocosms

To determine the chlorate exposure concentrations for the mesocosm study, we analyzed available water quality monitoring data for the wetland (monthly records) and effluent monitoring data (weekly records) for the period February-April 2004 (CELCO 2004, 2008). With this information, and for a period of 3 months, plants within a subset of 5 tanks of the mesocosm experiment were exposed to the maximum chlorate concentration of 0.52 mg/L recorded at the head of the wetland. At the same time, plants within another subset of 5 tanks were exposed to a slug of chlorate concentrations similar to those estimated for the Cruces River immediately downstream of CELCO's effluent discharge, based on effluent monitoring data (Table 1). This group of plants was therefore exposed to weekly concentrations of the maximum chlorate values of 1.6, 7.8, 4.6 and 0.9 mg/L, receiving only river water during the month prior and the month after exposure to chlorate. It is expected that significant natural attenuation of chlorate concentrations take place within the wetland due to mixing and sediment uptake and reduction. Hence, these concentrations based on effluent chlorate data must be regarded as conservative estimates. A fate and transport study would be needed to determine more realistic values.

Description of the Ecotoxicity Analysis

This analysis was performed in a wet laboratory facility at the Universidad de Concepción in a semi-static fashion, utilizing 5-L plastic buckets filled with water from the same lagoon (Lo Galindo in Concepción, Chile) where *E. densa* plants were obtained. Lo Galindo is a freshwater lagoon located within the city and was chosen as a source of water and specimens because its closeness and also because *E. densa* exists there in high abundance, so much that it has to be regularly removed in order to provide access to the shores. Following standard procedures described for aquatic primary producers affected by pollutants like SO_2 (Sha et al. 2010) or chlorate (i.e. Rosemarin et al. 1994; Scheerbaum 2003) we quantified the EC50 of chlorate for *E. densa* considering growth-related morphological parameters, including those previously used on similar studies (Palma et al. 2008). For 1 month we kept plants (3 individuals per container) exposed to different concentrations of chlorate. The chlorate source was sodium chlorate similar to that used by CELCO, and its concentration inside the containers was verified by ion chromatography. Nine pairs (replicates) of containers had different chlorate concentrations and 3 containers were designated as controls (water without chlorate). The tested chlorate concentrations were defined by a geometric series with a 3.2 separation factor in order to cover a

Table 2 Average (standard deviation) values for several physical and chemical variables measured inside the tanks of both chronic and acute experiments

	O_2(mg/l)	Conductivity (μS/cm^2)	pH	T (°C)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)
Chronic exposure to chlorate						
Treatment	6.7 (0.8)	32.0 (1.7)	7.5 (0.3)	19.8 (1.6)	0.18 (0.03)	0.56 (0.01)
Control	6.6 (0.7)	29.7 (1.4)	7.3 (0.5)	19.3 (1.8)	0.17 (0.02)	0.56 (0.01)
Acute exposure to chlorate						
Treatment	6.6 (0.8)	[a]73.2 (61.7)	7.6 (0.2)	20.2 (1.3)	0.19 (0.02)	0.56 (0.01)
Control	6.5 (0.8)	30.9 (1.0)	7.5 (0.1)	19.7 (1.4)	0.17 (0.01)	0.56 (0.01)

[a] Elevated value and variability due to chlorate addition during the second experimental week

broad range of concentrations (in mg/L): 1; 3.2; 10.2; 32.8; 104.9; 335.5; 1,073.7; 3,436.0 and 10,995.1.

For this experiment, we utilized the top 30 cm of young and healthy looking plants, without roots or lateral branches, obtained from Lo Galindo lagoon, which were acclimated for a period of 2 days. The whole array of tanks was set up in the open and only a mesh roof was placed on top in order to avoid excess sunlight and potential photo-oxidation. This precaution was taken since plants settled to the bottom of these small (30 cm deep) containers.

The principle behind the toxicity test is to compare the magnitude of the change (between the starting point and subsequent periods) of variables associated with the development of *E. densa*. The variables considered and measured at the beginning of the test, on December 8th, and then on December 23rd, 2010 and finally on January 8th, 2011, were: apical length (mm) and number of apical nodes. We utilized these variables based on previous results of mesocosm experiments (Palma et al. 2008) because they exhibited the most reliable information regarding growth rates. From the inhibition of apical growth rates we determined the EC50 level through the fit of a generic response curve. Additionally, we did a statistical determination of the lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) values. The LOEC corresponds to the lowest concentration at which chlorate has a statistically significant effect ($p<0.05$) compared with the control. The NOEC level is the tested concentration immediately below the LOEC level.

The evaluation of the toxicity response to chlorate (i.e. EC50) was performed with the statistical package Prism (GraphPad Software, Icn. Version 5.0b, 2009, La Jolla, California). The generic curve that describes the response of any parameter (in our case morphometric variables associated with the growth of *E. densa*) has an "S" shape, and the general model considered here is:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom})/(1 + 10\text{^}((\text{Log}_{EC50} - X) * \text{HillSlope}))$$

Where, Y is the dependent morphometric variable (i.e. apical length in mm), X is the concentration of chlorate (mg/L); Top is the maximum value of the morphometric variable; Bottom is the minimum value of the morphometric variable and HillSlope is the model's adjusting parameter associated with the slope of the curve.

For all the variables, the magnitude of the observed change was analyzed for two time intervals (T1-T0: difference between measurements recorded after 2 weeks and those at the beginning of the experiment, and T2-T0: difference between measurements recorded after 4 weeks and those at the beginning of the experiment). Prior to the analysis with the equation described above, the chlorate concentration values were log-transformed. All the data analysis was adjusted using least squares.

LOEC and NOEC thresholds were statistically determined using one-way ANOVA (after inspection of the data in order to assess normality of the data) and posthoc paired comparisons (Tukey test). For each temporal set (T1-T0 or T2-T0) the average values for each level of chlorate concentration were compared against the control values.

Results

Chronic Exposure to Chlorate for 12 Weeks

Mesocosm trials with plants maintained for 3 months under chronic chlorate levels (0.52 mg/L) as well as the control ones (no chlorate added) exhibited a general increase in the magnitude of the different morphometric variables considered (Fig. 2), which is reflected in the significant values of "time" as a factor in 5 of the 7 cases (Table 3). None of the variables showed a significant difference between chronic exposure and control. After a pairwise Bonferroni test, no statistically significant difference was detected (Fig. 2).

Acute Exposure to Chlorate for 5 Weeks

In concordance with the results of the chronic exposure results reported above in this analysis, we observed a general increase for all the morphometric variables considered (Fig. 3). None of the variables showed significant differences between plants exposed to acute levels of chlorate and the control, nor were significant the interactions between treatment and time (Table 4).

Chlorophyll-*a* Concentration in Experimental Plants

Chlorophyll-*a* was quantified in plants that were exposed to the experimental conditions described above (chronic, acute and control groups), and although slight, there was a difference in chlorophyll-*a* abundance among treatments (Chronic treatment: 1.106±0.498 mg/L; Acute: 1.224±0.182 mg/L; Control: 2.339±0.498 mg/L). The concentration of chlorophyll-*a* was greater in the control plants compared to the plants receiving a chronic level of chlorate ($F_{1,2}=4.295$, $P=0.049$, Dunnett post hoc test) but not compared to those under the acute levels of chlorate.

Ecotoxicological Experiments with Variable Chlorate Levels (1 Month)

Determination of the EC50

The EC50 is defined as the concentration of any substance (chlorate in this case) that causes an average response between the smallest (zero growth or 100 % inhibition) and the

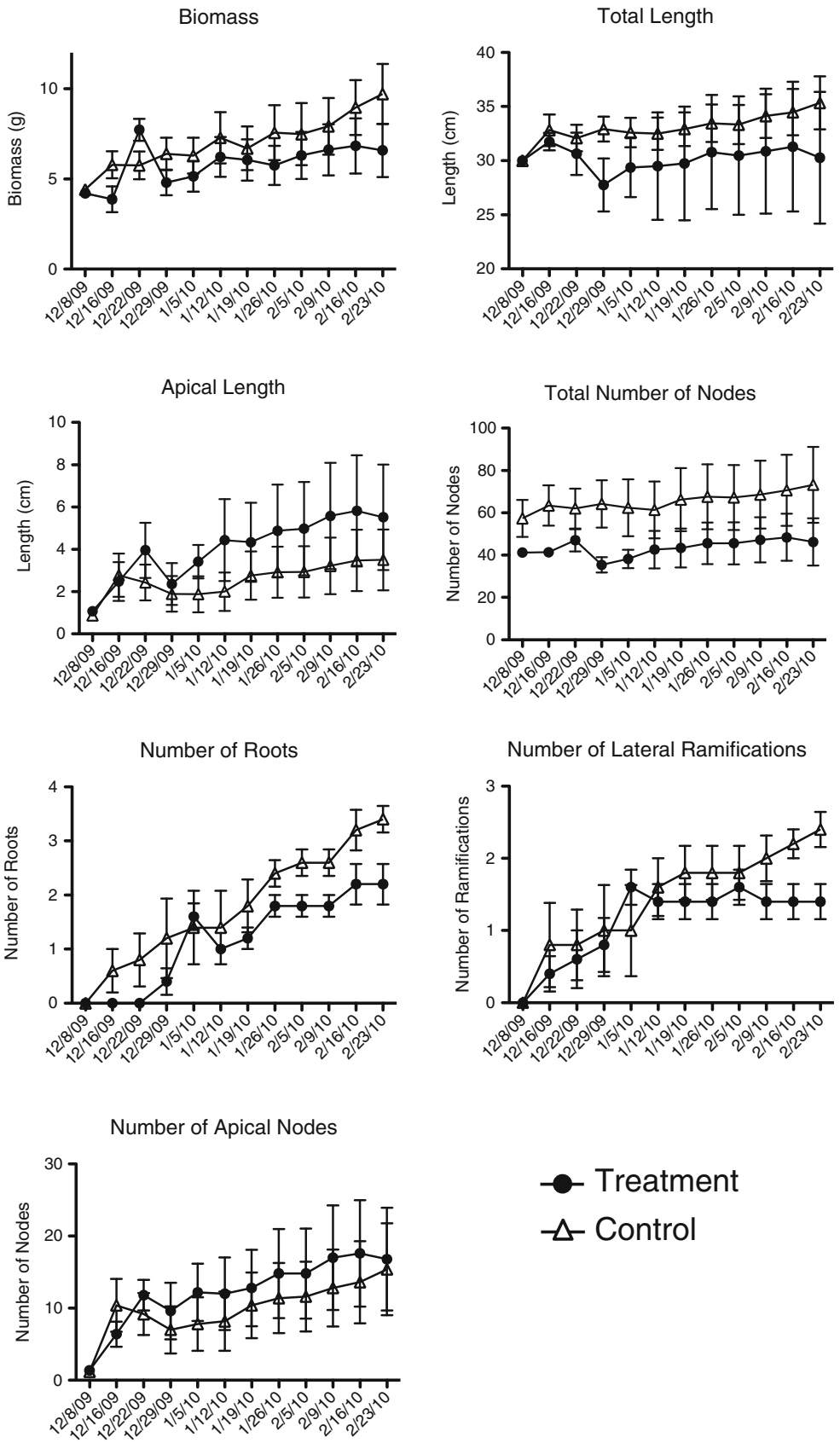

Fig. 2 Weekly variation (12 weeks) of different morphological variables of *Egeria densa* exposed to a chronic treatment of chlorate (0.52 mg/l). Error bars correspond to standard errors

Table 3 Results of the two-way repeated measures ANOVA comparing the growth (several parameters) of *Egeria densa* plants under a chronic dose of chlorate (0.52 mg/l) vs. the control situation (only Cruces River water)

Source of variability	df	MS	*F*	*p*
Biomass				
Treatment	1	41.8	0.6734	0.4356
Error	8	62.06	32.02	<0.0001
Time	11	13.24	6.83	<0.0001
Time × Treatment	11	3.868	1.995	0.0381
Error	88	1.938		
Total length				
Treatment	1	242	0.4929	0.5026
Error	8	490.9	30.8	<0.0001
Time	11	8.896	0.5581	0.8574
Time × Treatment	11	5.464	0.3428	0.9733
Error	88	15.94		
Apical Length				
Treatment	1	68.68	0.8221	0.391
Error	8	83.53	17.27	<0.0001
Time	11	11.81	2.443	0.0104
Time × Treatment	11	2.072	0.4284	0.9395
Error	88	4.836		
Number of nodes				
Treatment	1	14301	2.148	0.1809
Error	8	6658	69.58	<0.0001
Time	11	135.5	1.417	0.1797
Time × Treatment	11	38.6	0.4034	0.9511
Error	88	95.68		
Number of roots				
Treatment	1	11.41	3.287	0.1074
Error	8	3.471	9.246	<0.0001
Time	11	9.039	24.08	<0.0001
Time × Treatment	11	0.3902	1.039	0.4196
Error	88	0.3754		
Number of ramifications				
Treatment	1	3.008	0.6171	0.4548
Error	8	4.875	20.43	<0.0001
Time	11	3.493	14.64	<0.0001
Time × Treatment	11	0.4083	1.711	0.0838
Error	88	0.2386		
Number of apical nodes				
Treatment	1	165.7	0.1608	0.6989
Error	8	1031	30.9	<0.0001
Time	11	165.5	4.964	<0.0001
Time × Treatment	11	13.69	0.4106	0.9479
Error	88	33.35		

greatest observed value for a chosen variable (maximum growth or 0 % inhibition) based on the model considered here. The EC50 values were recorded 4 weeks after beginning the exposure of the plants to increasing concentrations of chlorate (Fig. 4).

In our assay, an EC50 value of 1037 mg/L (the only significant different value with a determination coefficient of 0.69) was detected, but only for the inhibition of apical growth by measuring apical length. It is important to notice that even though the model only detected EC50 values after 4 experimental weeks, the graphs (Fig. 4) show a clear decay of the fitting curves toward the end of the tested concentrations, and mainly for the T2-T0 exposure interval.

Determination of the LOEC

The variables quantified in all plants in two opportunities (2 and 4 weeks after the beginning of the experiment) exhibited a response to the addition of chlorate (Fig. 4). The change in size (apical length and number of apical nodes) was always positive, after 2 and after 4 weeks, although the later change in size was more noticeable. Compared to control plants, those receiving chlorate started showing a significant decrease in growth rate at the highest chlorate concentrations utilized here. The significance of the differences between plants exposed to different chlorate concentrations versus the controls allowed us the determination of the chlorate concentration at which *E. densa* started showing signs of growth inhibition (i.e. the lowest observed effect concentration, LOEC). For the two parameters considered, significant differences were first and only observed in plants after 2 weeks of exposure to 104.9 mg/l ($P<0.01$). Hence, the NOEC (no observable effects concentration) is 32.8 mg/l.

Discussion

Although difficult, particularly because receiving aquatic systems are often exposed to multiple stressors (Lowell et al. 2000), the ability to understand the relative effect of individual factors represents a step forward toward improving our ability to make valid ecologically-based predictions and to contribute with regulatory guidelines (Wiegner et al. 2005). Mesocosms have been successfully utilized in order to achieve those goals in river systems (i.e. Culp et al. 1996; Dubé et al. 2002). The relatively large size and flow-through characteristics of this experimental approach allows controlling exposure conditions while maintaining environmental realism (Dubé 2004). Hence, the use of mesocosms allows the isolation of factors that are hypothesized to have negative effects on identified ecosystem endpoints.

While other mesocosm studies have considered the effect of chlorate on diatoms and other freshwater microalgae assemblages for periods lasting less than 1 month and finding NOEC between 0 mg/L and 300 mg/L of chlorate

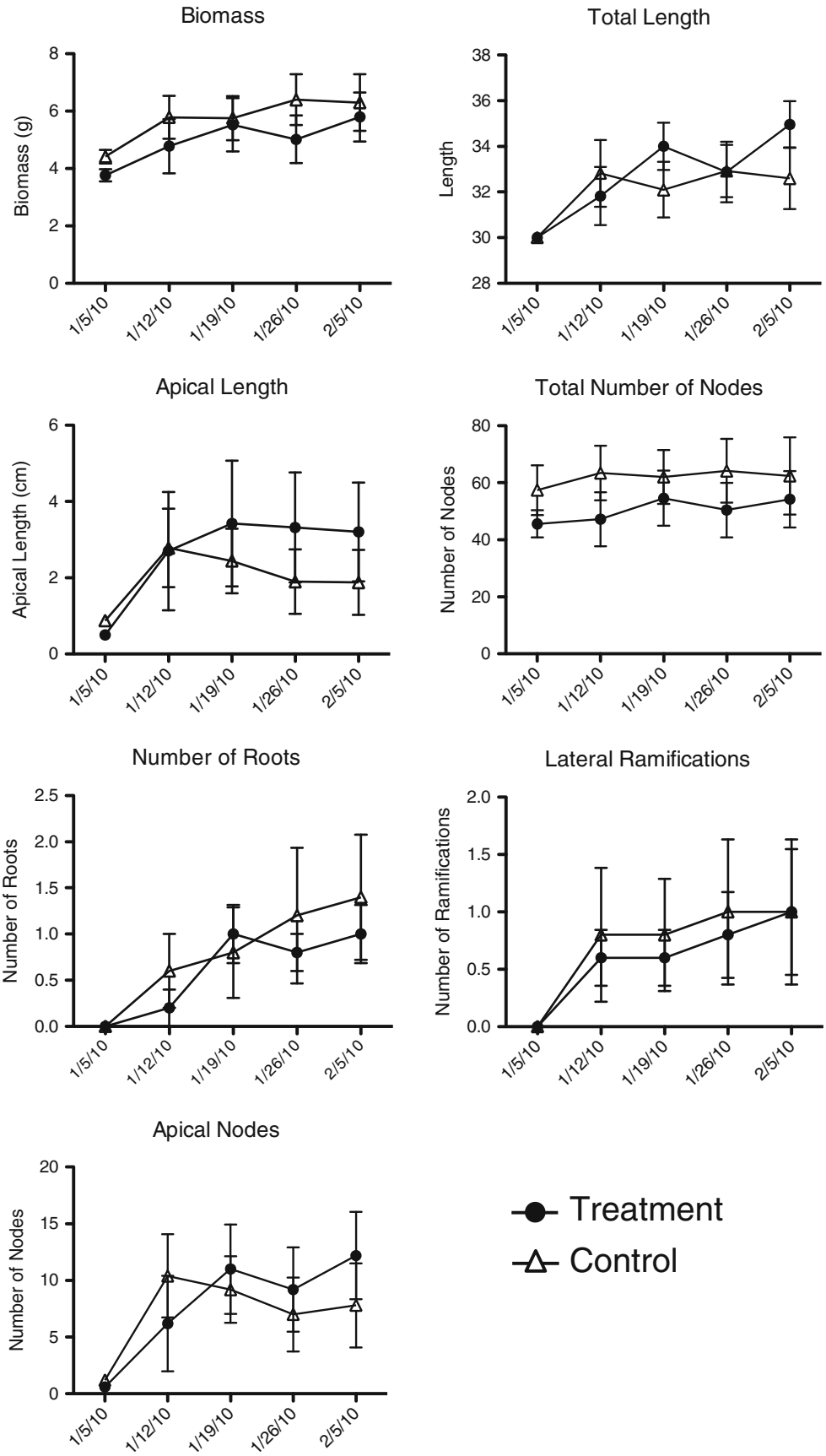

Fig. 3 Weekly variation (4 weeks) of different morphological variables of *Egeria densa* exposed to an acute concentration regime of chlorate (1.6, 7.8, 4.6, 0.9 mg/L every week, respectively). Error bars correspond to standard errors

Table 4 Results of the two-way repeated measures ANOVA for comparing the growth (several parameters) of *Egeria densa* plants under an acute dose of chlorate (1.6, 7.8, 4.6, 0.9 mg/L every week, respectively) vs. the control situation (only Cruces River water)

Source of variability	df	MS	*F*	*p*
Biomass				
Treatment	1	7.099	0.5696	0.4721
Error	8	12.46		
Time	4	5.726	7.82	0.0002
Time × Treatment	4	0.5024	0.6861	0.6069
Error	32	0.7322		
Total length				
Treatment	1	5.184	0.2306	0.644
Error	8	22.49		
Time	4	20.86	10.25	<0.0001
Time × Treatment	4	5.067	2.489	0.0629
Error	32	2.036		
Apical length				
Treatment	1	5.314	0.2548	0.6273
Error	8	20.85		
Time	4	8.341	3.517	0.0173
Time × Treatment	4	1.715	0.7234	0.5824
Error	32	2.371		
Number of nodes				
Treatment	1	1647	0.7933	0.3991
Error	8	2077		
Time	4	82.28	1.028	0.4078
Time × Treatment	4	34.48	0.4308	0.7853
Error	32	80.03		
Number of roots				
Treatment	1	0.5	0.1754	0.6863
Error	8	2.85		
Time	4	2.4	7.111	0.0003
Time × Treatment	4	0.2	0.5926	0.6705
Error	32	0.3375		
Number of ramifications				
Treatment	1	0.18	0.05099	0.827
Error	8	3.53		
Time	4	1.53	4.636	0.0046
Time × Treatment	4	0.03	0.09091	0.9847
Error	32	0.33		
Number of apical nodes				
Treatment	1	6.48	0.03608	0.8541
Error	8	179.6		
Time	4	143.9	6.288	0.0008
Time × Treatment	4	26.78	1.17	0.3424
Error	32	22.89		

(Perrin 1992; Perrin and Bothwell 1992), our study represents the first attempt with a freshwater macrophyte under experimental conditions lasting over 1 month, similar to the long-term studies with marine brown algae (Rosemarin et al. 1994). We demonstrate that for both a chronic exposure for 3 months and an acute exposure to chlorate for 1 month, there was no negative effect of this compound on most of the growth-related parameters considered in *Egeria densa*. On the other hand, there was no lethal effect on plants exposed to any of the doses of chlorate utilized here, regardless of having detected a lower increase in biomass and fewer numbers of nodes in the acute treatment. Ultimately this could be expected and is consistent with the much higher EC50 value of 1037 mg/L obtained in this study.

Both mesocosm treatments were designed to represent extreme exposure scenarios in the wetland, and not entirely realistic ones, with the purpose of discarding any lethal

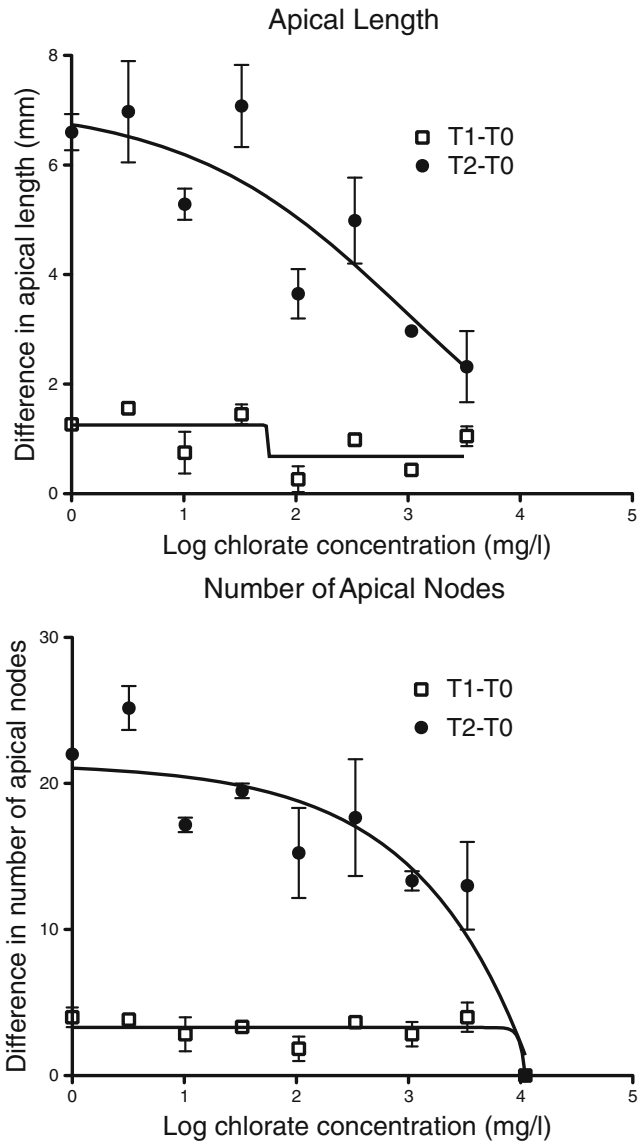

Fig. 4 Fitted curves for the determination of the EC50 on differences in apical length and the number of apical nodes of *Egeria densa* plants between sampling times T1-T0 and T2-T0

effects, that could have produced the massive disappearance of *E. densa* in 2004. On the one hand, the chronic treatment had a duration of 3 months while the actual measured value of 0.52 mg/L is representative of only 1 month in the Cruces River and, on the other, the acute exposure did not take into account natural attenuation of the chlorate slug in the wetland due to mixing and sediment uptake. Hence, the sublethal effects observed in some variables are likely to be an overstatement of what could have occurred given the 2004 conditions in the wetland. More realistic exposure scenarios could be determined by a chlorate fate and transport study.

In spite of the negative effect of chlorate on brown algae in coastal ecosystems in the Baltic Sea (Lehtinen et al. 1988; van Wijk and Hutchinson 1995), there is limited evidence of toxicity for aquatic angiosperms like *E. densa* (e.g., freshwater aquatic plant *Lemna minor* (Scheerbaum 2003)). Thus, it is not valid to just extrapolate the negative effect of chlorate on this algal species to the macrophyte *E. densa*. Reported chlorate threshold toxicity values available for aquatic plants are elevated (EC50/7 days=105 mg/L for growth rates of *Lemna minor* (Scheerbaum 2003)). Additionally, in order to evaluate the risk of chlorate in aquatic ecosystems, van Wijk and Hutchinson (1995) reviewed the toxicity of chlorate to aquatic organisms. The geometric means of the E(L)C50 (toxic or lethal concentration for 50 % of the population) values for marine as well as for freshwater species were: microorganisms = 39 mg/L; micro algae = 560 mg/L; invertebrates = 2,442 mg/L and fish = 3,815 mg/L. These authors concluded that chlorate is non-toxic (acute toxicity or lethal concentrations for 50 % of the population > 100 mg/L) for most of marine and freshwater species examined. Chlorate was only toxic (acute toxicity <0.1 mg/L) for certain brown algal species (*Macrocystis* and *Fucus*), which do not occur in the Cruces River. The most relevant reference for a possible toxic effect of chlorate on *E. densa* is the EC50 value of 105 mg/L found in *Lemna minor* (Scheerbaum 2003). Comparatively, the maximum concentration of chlorate estimated for the Cruces River upstream of the wetland in 2004 was 7.8 mg/L, which is expected to be attenuated within the wetland due to mixing of river and wetland waters. The EC50 for chlorate in our study was 1,037 mg/L, the LOEC 104.9 mg/L and the NOEC 32.8 mg/L. The rather high acute toxicity threshold (EC50) of chlorate for the apical growth detected in this study is more than 3 orders of magnitude higher than the maximum chlorate concentrations measured and estimated for the Cruces River wetland. These toxicity thresholds constitute evidence in favor of rejecting the hypothesis suggesting chlorate as the agent behind the environmental changes that affected this ecosystem. The EC50 values for *E. densa* were also significantly greater than the values determined for *Lemna minor* and for marine and freshwater algae.

It is assumed that chlorate is taken-up by the plant cells through the system that assimilates nitrate and then is converted to chlorite, the supposedly toxic compound. Hence, it is expected then that the toxic effect of chlorate be expressed under environmental conditions of low nitrate levels. For example, Stauber (1998) found that nitrate inhibits the uptake of chlorate in species of marine micro-algae (*Nitzschia closterium* and *Dunaliella tertiolecta*). Previous studies in the Cruces River between 1995 and 1996 indicate that nitrate levels in the Cruces River are relatively high (annual average of 268.9 μg/L) in comparison with other water systems in southern Chile. Furthermore, nitrate concentrations are greater during the winter months, likely due to greater uptake by primary producers during summer months (Campos 1997). On the other hand, there is evidence that *E. densa* shows preference for ammonia as nitrogen source and that this nitrogen uptake takes place mainly by diffusion directly from the water and not through the roots (Feijoó et al. 2002). There is also evidence that phosphorous in the Cruces River basin is comparatively more limiting than nitrogen (Yarrow et al. 2009). Hence, our results showing no significant negative effect of chlorate on *E. densa* plants under experimental conditions agree with the previously described scenario whereas the toxic mechanism for chlorate toxicity is not favored in this Ramsar wetland. Furthermore, recent field observations (unpublished data) show that *E. densa* (as well as large numbers of black necked swans) is returning to the system while the pulp mill plant is still operating and discharging effluents into the Cruces River. Future research should focus on a better understanding of the ecophysiology of this important aquatic macrophyte as well as on implementing a thorough and long-lasting environmental monitoring program in this wetland system.

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