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Phytoremediation potential of *Ulva ohnoi* (Chlorophyta): Influence of temperature and salinity on the uptake efficiency and toxicity of cadmium

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ABSTRACT

Ulva ohnoi is a green macroalga with fast growth and high rates of nitrogen and phosphorus absorption. Recently, this species has been recorded in several places with record green tide formation in some of them. Using molecular tools, we herein report the first occurrence of this species in Brazil and demonstrate its potential for phytoremediation in typical environmental concentrations of Cd (0.625–15 μg L^{-1}). Similarly, the effects of physicochemical parameters (salinity and temperature) on the toxicity and uptake efficiency of this species were evaluated. Molecular analysis of two sequences (1141 bp) obtained corroborates another 34 sequences for *U. ohnoi* obtained from GenBank. The addition of Cd in the medium affected photosynthetic parameters and reduced growth rate. *U. ohnoi* showed resistance to Cd when cultivated at 18 °C, S15 and 18–25 °C, S35, at concentrations between 0.625 and 2.5 μg. L^{-1} of Cd; yet, positive growth rate was maintained. Dose-dependent accumulation was observed in all combinations of factors used with a maximum value of 4.20 μg Cd per gram of dry seaweed at 15 μg. L^{-1} of Cd at 18 °C and S35. Maximum value of the concentration factor was 81.3 ± 1.1% of Cd added at the concentration of 0.625 μg. L^{-1} to S15 and 18 °C. Our results demonstrate the potential of using *U. ohnoi* in the phytoremediation of Cd in saltwater or brackish water.

1. Introduction

The increasing human population in recent decades has, in turn, caused the intensification of industrial activities and correspondingly increased demand for raw materials (Volesky, 2001). As a consequence, many xenobiotic compounds have been mobilized and released in high concentrations into the environment, causing changes in physical, chemical and biological attributes (Martins et al., 2012; Scherner et al., 2018, 2013). Among these compounds, dissolved free trace metal ions cause many negative environmental effects and bioaccumulation in the ecosystem (Barwick and Maher, 2003; Caruso et al., 2011), including lead (Pb), copper (Cu), cadmium (Cd), zinc (Zn), and nickel (Ni), as the most frequent metal pollutants (Duruibe et al., 2007). Among them, Cd is the only metal which has no reported biological function, except as a cofactor for carbonic anhydrase in the marine diatom *Thalassiosira weissflogii* (Grunow) G.Fryxell & Hasle under low zinc conditions (Lane and Morel, 2000). Its availability in the environment occurs frequently in small concentrations by weathering or in larger concentrations in relation to mining and mineral processing, the application of pesticides and battery manufacturing (Babich and Stotzky, 1978). In aquatic environments, Cd is freely transported and absorbed by organisms owing to the high solubility and similarity to essential metals (Fe^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Ca^{2+}) (Peters et al., 1997). In photosynthetic marine organisms, especially macroalgae, the effects of Cd have been extensively studied, mainly those associated with physiology and ultrastructure (Collén et al., 2003; Costa et al., 2016; Markham et al., 1980; Webster and Gadd, 1996, 1992; Wu et al., 2009). Most of these studies have focused on the evaluation of the isolated effects of a metal, overlooking interactions with key environmental factors which can change toxicity and bioaccumulation capacity (Scherner et al., 2018).

Variable environmental factors, such as temperature and salinity, interfere with different biological processes related to the absorption

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and/or incorporation of metals (Lobban and Harrison, 1994; Scherner et al., 2018). Temperature influences absorption rates as a result of changes in metabolic demands and membranes properties, such as permeability, fluidity and membrane phase state (Eggert, 2012). Variations in salinity influence ionic concentrations, nutrient uptake, osmotic regulation and physiological processes, leading to biochemical and structural changes (Karsten, 2012). In the environment, variations in salinity can cause changes in pH and increase the bioavailability of metal (Munda, 1984). Studies that report on the responses of algae exposed to isolated environmental factors are common and contribute to improving the disposal of industrial waste (Dawes et al., 1999; Floreto et al., 1993; Macler, 1988; Mantri et al., 2011; Yokoya and Oliveira, 1992). However, studies reporting on the interactions between environmental factors and contaminants, such as trace metals, are still scarce (Lee and Wang, 2001; Scherner et al., 2018). Multifactorial designs are laborious, but necessary, to improve realistic scenarios regarding applications of bioremediation tools in wide environmental gradients related to time (seasonality) or space (latitude) (Scherner et al., 2018).

Macroalgae are among the most practical and economical options to remove pollutants from contaminated areas. Several studies using biomass have been carried out showing potential beneficial effects (Kang and Sui, 2010; Lee and Wang, 2001; Sode et al., 2013; Wu et al., 2018). Nevertheless, little is known about how environmental factors affect the absorption of contaminants (Choi et al., 2010; Fan et al., 2014), especially in the case of trace metals. Reduction of salinity was related to an increase of more than 70% in the absorption of Cd (Felix et al., 2014). Lee and Wang (2001) obtained a rise of 137% in Cd content in *Ulva fasciata* Delile when the concentration of nitrate in the medium was increased. Among the species studied, those belonging to the genus *Ulva* Linnaeus are noteworthy for their high absorption rate and rapid growth, which, together with their tolerance to a wide range of environmental conditions, make them attractive candidates for bioremediation (Angell et al., 2015).

The genus *Ulva* (Chlorophyta) is cosmopolitan and common in all oceans and estuaries (Guiry and Guiry, 2018). Some species of *Ulva* have high nitrogen (N) and phosphorus (P) absorption capacity and are responsible for the formation of green tides (Melton et al., 2016a). Studies have shown that green tides are triggered by physicochemical factors, including temperature, light, salinity and N and P concentration, and that these green tides may have negative effects on ecosystems, if neglected (Li et al., 2016; Smetacek and Zingone, 2013), whereas they can promote benefits, environmental goods and services if managed correctly (Wu et al., 2018). Further studies can improve our understanding of the factors related to blooms, productivity, and metal absorption in bioremediation systems. This fast growing abilities has fostered the use of *Ulva* species in wastewater treatment (Al-Hafedh et al., 2015; Copertino et al., 2009; Tsagkamilis et al., 2010). Additionally, the cell walls of *Ulva* species contain polysaccharides with negatively charged sulfate and carboxyl groups, giving these structures a potential binding site for different trace metals, such as Cd (Jaulneau et al., 2010). Moreover, the metals can be internalized and found in all cellular organelles (Webster and Gadd, 1996).

Nowadays, there is a need to develop technologies to reduce Cd levels to acceptable limits in a cost-effective and environmentally friendly way. Prospecting for biodiverse species and manipulating environmental conditions are fundamental for the development of phytoremediation processes in a way that can accommodate the needs of particular localities. During a survey in the archipelago of Fernando de Noronha, *Ulva* specimens were collected and molecularly identified as *Ulva ohnoi* M. Hiraoka & S. Shimada. Several studies have been carried out with this green tide-forming species, demonstrating its potential use in bioremediation of aquaculture effluents (Lawton et al., 2013; Masaló et al., 2016), although its potential for trace metal uptake, such as cadmium, remains to be elucidated. In this context, the present work aimed to evaluate the effects of environmental factors (temperature and salinity) on the ability of *U. ohnoi* to uptake Cd, using physiological descriptors to understand the relationship between the factors used.

2. Material and methods

2.1. Algal material and culture conditions

The algal samples were collected at Buraco da Raquel (03°50′4.95″S–32°23′52.06″W) in the archipelago of Fernando de Noronha, 360 km off the northeast coast of Brazil (03°54′S–32°25′W). The archipelago has a very low level of urbanization and virtually no industrial activity. The climate is tropical with an average seawater surface temperature of 28 °C throughout the year (Sampaio de Souza et al., 2013).

U. ohnoi specimens (5 g) were collected from the rocky shores in March 2015 and transported in Falcon tubes, kept in dark and ambient temperature, to LAFIC-UFSC (Laboratory of Phycology, Federal University of Santa Catarina, Florianopolis, Santa Catarina, Brazil). The seaweed was cleaned to remove sand and epiphytes using paint brushes and sterilized seawater. Parts of the algal thalli were separated for molecular identification. The seawater was sterilized by mechanical filtering (5 and 10 μm) and exposed, in continuous flow, to ultraviolet C irradiation. Unialgal cultures were maintained with von Stoch solution enriched sterilized seawater (8 mL L^{-1}) at 25 ± 1 °C, salinity 35, constant aeration, 120 ± 10 μmol photons m^{-2} s^{-1} (4 fluorescent lamps, Osram 30 W DayLight) and 12:12 light:dark cycle. Culture medium was replaced weekly, and the seaweeds were weighed to maintain a density of 5 g (wet weight) per liter. Irradiance was measured with a LI-COR LI-1400 DataLogger and UnderWater sensor UWQ8815.

2.2. Molecular studies

2.2.1. DNA extraction and amplification

For molecular identification, about 10 mg of tissue were used to extract DNA from two sampled individuals. The samples were placed in a 1.5 mL Eppendorf tube, frozen with liquid nitrogen and ground for 2 min with a plastic pestle. The DNA content was extracted using a commercial kit [NucleoSpin® Plant II, Macherey-Nagel, Düren, Germany], following the manufacturer's instructions. The large subunit of the plastid-encoded Ribulose Bisphosphate Carboxylase-Oxygenase (RuBisCO) gene region (rbcL) was amplified in a polymerase chain reaction (PCR) using published primer pairs RH1 and 1385r (Manhart, 1994) and cycling conditions described in Loughnane et al. (2008). After amplification, the purification of the PCR products was performed using PEG 8000 (Polyethylene Glycol 8000) (Lis and Schleif, 1975).

2.2.2. Sequencing

Sequencing of the PCR products was done by the chain termination method (Sanger et al., 1977) with a Big Dye Terminator v 3.1 kit (Thermo Scientific, Carlsbad, CA, USA), following the protocol specified by the supplier. The sequential reaction of the resulting cycle was purified with etOH/EDTA precipitation and sequenced in DNA analyzers (3500 XL or ABI 3730, Applied Biosystems, Thermo Fisher Scientific) at the Laboratory of Plant Physiology and Developmental Physiology, Federal University of Santa Catarina. The resulting chromatograms were assembled using Sequencing Analysis software v6.0 (Thermo Fisher Scientific).

2.2.3. Alignment

Two rbcL sequences were generated with 1141 bp. Consensus sequences were assembled from direct and inverse readings. Quality analysis and editing of the consensus sequences were performed in Geneious, version R9 (http://www.geneious.com, Kearse et al., 2012). The concordance DNA sequences were aligned using the Muscle method in MEGA software 7.0.26 (Tamura et al., 2011). Other (44) rbcL sequences from the genus *Ulva* were downloaded from GenBank, and a

sequence of *Umbraulva olivascens* M. J. Wynne & G. Furnari was added to the alignment as an external group.

2.2.4. Phylogenetic analysis

Phylogenetic analyses were performed using the Maximum Likelihood (ML) methods with a GTR + G model. ML analyses were executed by the RxML-HPC2 program using 'The CIPRES Science Gateway V.3.3' online server (Miller et al., 2010) with 1000 replicates. Support for resulting relationships was estimated by 1000 bootstrap replications.

2.3. Experimental design and procedures

A trifactorial design was set to evaluate the combined effects of temperature, salinity and Cd on the physiology and uptake in *U. ohnoi*. After five months of cultivation in the laboratory, fragments of *U. ohnoi* were selected (0.50 ± 0.02 g FW per flask) and incubated in three temperatures (18 ± 1, 25 ± 1 and 28 ± 1 °C), two salinities (S15 and S35), and five Cd concentrations (0, 0.625, 2.5, 5 and 15 μg. L^{-1}), totaling 30 treatments. Three replicates for treatments were used for each combination. These temperatures represent values found along the Brazilian coast, and these salinities represent values common in mangroves and the coastal ocean (NOAA, 2015; Pagliosa et al., 2006). The investigated temperatures were obtained using a gradient table described by Oliveira et al. (1995), and the salinity levels were obtained by diluting seawater with distilled water (Oliveira et al., 1995). The Cd concentrations used represent a gradient of values found along the Brazilian coast and values permitted by Brazilian legislation for marine and brackish waters (CONAMA, 2005; Ferreira et al., 2004). These concentrations were achieved by dissolving $CdCl_2$ (CAS 10108-64-2) into seawater. Treatments were maintained for 8 days under the same culture conditions, except temperature and salinity (used according to the treatment), and von Stoch solution (prepared without the addition of EDTA). The pH of the water was monitored and kept constant at 8.7 ± 0.6.

2.4. Bleaching degree and growth rates

The degree of algal bleaching was determined by photographing different thalli at the end of the experiment using the Canon G12 camera with the same settings (ISO – 100, diaphragm value – f/8, capture speed – 1/80 s) and lighting (back light – VitraLux E27). The images generated were analyzed with ImageJ, v.1.8.0, followed by calculating the percentage (%) of bleached area in relation the total area of *U. ohnoi* thalli. Fresh biomass was recorded with an analytical balance (BioPrecisa FA-2104N) at the beginning and end of the experiment. Growth rate was calculated as $GR\ (\%\ day^{-1}) = [(W_t/W_i)^{1/t}-1]\times 100$, where W_i = initial fresh biomass, W_t = fresh biomass after 8 days, and t = experimental time in days.

2.5. Photosynthetic parameters

Photosynthetic parameters were obtained by in vivo chlorophyll *a* fluorescence analysis using a Pulse Amplitude Modulation Fluorometer (WaterPAM, Walz, Effeltrich, Germany). Light acclimated algae (120 ± 10 μmol photons.m^{-2}. s^{-1}) were submitted to three (pseudoreplicate) saturating pulses per flask (approximately 10000 μmol photons. m^{-2}. s^{-1} for 0.8 s) taken at different parts of the thallus. Effective quantum yield ($\Delta F/F_m'$) was calculated as $\Delta F/F_m' = \frac{(F_m' - F_t)}{F_m'}$, where F_t is the current steady-state fluorescence in light-adapted sample, and F_m' is the maximum fluorescence from the same sample after a saturating white light pulse. The $\Delta F/F_m'$ data acquired in this way were used to calculate the Absolute Electron Transport Rate (ETR_{abs}) as $ETR_{abs} = \Delta F/F_m' \times E \times A \times 0.5$, where E is the actinic irradiance, A is the absorptance and 0.5 is the fraction of chlorophyll associated with photosystem II in green macroalgae (Grzymski et al., 1997). The absorptance was obtained as described by Korbee et al. (2005). The samples were acclimated in the dark for 30 min, allowing measurement of basal fluorescence (F_0). Afterwards, samples were exposed to nine increasing actinic irradiances (0, 170, 250, 330, 450, 570, 680, 825 and 1200 μmol photons m^{-2} s^{-1}) for 10 s. Saturating white light pulses were applied between each irradiated exposure. After the first pulse, the maximum fluorescence (F_m) value was obtained, and the maximum quantum yield (F_v/F_m) was calculated as $F_v/F_m = (F_m - F_0)/F_m$. The data acquired after the second pulse were used to calculate $\Delta F/F_m'$ and then ETR_{abs} for each actinic irradiation, allowing the construction of ETR_{abs} x irradiance PAR_{abs} curves. Three replicates were sampled for the analysis of photosynthetic parameters.

2.6. Cadmium uptake

Cd concentration was determined as described by Schneider et al. (2017). About 40 mg of dry algae (48 h in 60 °C) were weighed and placed in a polypropylene flask to which were added 300 μL of HNO_3 and 300 μL of H_2O_2, followed by filling the flask up to 2 mL with deionized water (DI). The samples were then submitted to a thermostatic bath at 80 °C for 15 min. After cooling, the samples were centrifuged for 6 min at 4000 rpm, and the supernatant was used for further analysis in a high-resolution continuum source atomic absorption spectrometer model CONTRAA 600 (Analytik Jena, Jena, Germany). The temperature program for an Ir-coated graphite tube employed pyrolysis and atomization temperatures of 600 °C and 1300 °C, respectively. The instrumental calibration was carried out with aqueous solution in a range of 0.5 μg L^{-1} to 10 μg L^{-1} with limits of detection and quantification of 7.5 ng g^{-1} and 25 ng g^{-1}, respectively. The data obtained were expressed as Cd uptake and used to calculate the concentration factor as $(CF) = ([Cd]_{biomass}/[Cd]_{water})x100$, where $[Cd]_{biomass}$ is the concentration of Cd found in the biomass of *U. ohnoi*, and $[Cd]_{water}$ is the concentration in the Cd water used in the treatments.

2.7. Statistics

Effects of temperature, salinity and Cd concentration on *U. ohnoi* were analyzed by factorial ANOVA. Analysis of normality and homogeneity of the variances were performed to verify if the assumptions were met. When significant differences were observed ($p < 0.05$), the Newman–Keuls post hoc multiple comparison test was applied. Pearson correlation analysis was conducted with significance considered as $p < 0.05$. Statistical analyses were performed in STATISTICA 10 software (StatSoft, Inc. 2011).

3. Results

3.1. Maintenance in the laboratory - Pre-experiment

During pre-experimentation cultivation, *U. ohnoi* grew steadily with mean GR of 12 ± 3% day^{-1} and did not show epiphytic growth. Reproductive events were observed with release of biflagellate cells. No formation of new individuals was observed, indicating that the individuals used in this experiment were female or male gametophytes.

3.2. Molecular identification

U. ohnoi was found on an oceanic island in the tropical zone (annual mean seawater temperature of 28 °C), with oligotrophic characteristics (Sampaio de Souza et al., 2013), fixed to rocky substratum with a few dispersed individuals with a maximum length of 4 cm.

Alignment of two rbcL DNA sequences (1141 bp) obtained from the samples showed a monophyletic clade in a phylogenetic tree with the sequences found in GenBank for *U. ohnoi* (Fig. 1). Sequences obtained were identical to those published for the Hawaiian Islands (O'Kelly

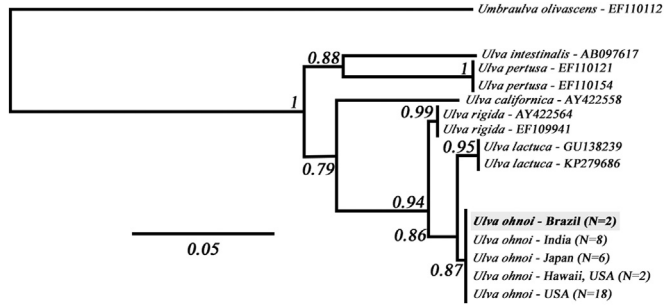

Fig. 1. Maximum likelihood phylogenetic tree run with 1000 bootstraps based on 1141 bp of the rbcL molecular marker. Other samples with rbcL sequences identical to the *Ulva ohnoi* sequences from Brazil (U7FNPE; U8FNPE) include the following GenBank Accession numbers: India (KP279696; KP279697; KP279698; KP279699; KP279700; KP279702; KP279703; KP279704 - Kazi et al., 2016); Japan (AB116035; AB116036; AB116037; AB116038; AB116039; AB116040 - Hiraoka et al., 2004b); Hawaii, USA (GU138241; GU138250 - O'Kelly et al., 2010) and USA (KU561279; KU561280; KU561282; KU561283; KU561285; KU561286; KU561287; KU561288; KU561289; KU561290; KU561291; KU561292; KU561293; KU561294; KU561295; KU561296; KU561297; KU561299 - Melton et al., 2016a).

et al., 2010), India (Kazi et al., 2016), USA (Melton et al., 2016a) and Japan, including the sequence of the type specimen (Hiraoka et al., 2004b) (Fig. 1).

3.3. Bleaching degree and growth rate

Effects of treatments (salinity, temperature and Cd concentration) on the bleaching degree of *U. ohnoi* are shown in Table 1. Factorial ANOVA indicates significant differences in bleaching degree for temperature, salinity, and Cd treatments; significant interaction among the treatments was also found (Table 2). Without the addition of Cd, samples at S15 presented a higher degree of bleaching than samples at S35 (12.41 ± 2.37% and 1.46 ± 0.95%, respectively). At S15, bleaching was higher at 25 °C (20.73 ± 1.01%), followed by 28 °C (13.73 ± 4.40%) and 18 °C (2.76 ± 1.69%). For S35, bleaching showed no significant differences among temperatures in Newman-Keuls post hoc test (Table 1). The addition of Cd increased bleaching for all temperatures at S15 (Table 1). The effect was more impressive (above 50% for the first concentration of Cd used) at 25 and 28 °C and gradual at 18 °C (above 50% with application 2.5 μg L^{-1} or higher). For S35, the effect of Cd on bleaching was only observed at 25 °C and 15 μg L^{-1} (9.50 ± 0.52%) and at 28 °C and 5 and 15 μg. L^{-1} (9.69 ± 2.91% and 45.39 ± 5.80%, respectively) (Table 1).

Growth rates of *U. ohnoi* were significantly influenced by temperature, Cd and salinity treatments, and significant interaction among the treatments was also found (Table 2). The highest GRs were found at 18 °C in S35 and S15 (5.57 ± 0.45 and 3.18 ± 0.52% day^{-1}, respectively), followed by 25 °C in S35 and S15 (4.99 ± 0.73 and 2.27 ± 0.54% day^{-1}, respectively). The decrease in growth rate was more pronounced in treatments with Cd addition where negative values (loss of biomass) were observed at all temperatures (Table 1). For 18 °C at S15, and 18 and 25 °C with S35, the GR was negative only when the Cd concentration was equal to, or greater than, 5 μg L^{-1} (Table 1).

3.4. Photosynthetic parameters

U. ohnoi cultivated under different salinities, temperatures and Cd concentrations had significantly different photosynthetic parameters and significant interactions among factors (Table 2).

For absolute electron transport rate (ETR_{abs}), the Newman-Keuls test showed that Cd significantly affected the treatments of 18 °C, S15, and

Table 1
Values of Bleaching Degree, Growth Rate (GR), Absolute Electron Transport Rate (ETR_{abs}), Maximum Quantum Yield (F_v/F_m), Cadmium Uptake (Cd uptake) and Concentration Factor (CF) of *Ulva ohnoi* exposed to two salinities (15 and 35), three temperatures (18, 25 and 28 °C) and five concentrations of Cd (0, 0.625, 2.5, 5 and 15 μg. L^{-1}) after 8 days. Values represent mean ± SD for N = 3. Different letters indicate significant differences according to trifactorial ANOVA and Newman-Keuls post hoc test ($p \leq 0.05$).

Salinity	Temperature	Cadmium (μg L^{-1})	Bleaching Degree (%)	GR (% day^{-1})	ETR_{abs} (μmol e^- m^{-2} s^{-1})	F_v/F_m	Cadmium Uptake (μg g^{-1} DW)	Concentration Factor (%)
S15	28 °C	0	13.73 ± 4.4b	1.23 ± 0.1ij	5.49 ± 0.35bcde	0.598 ± 0.03kl	0 ± 0abcd	0 ± 0a
		0.625	65.84 ± 6.02fg	− 1.75 ± 0.2abc	8.61 ± 0.56fghij	0.536 ± 0.055jkl	0.53 ± 0.16efgh	70.28 ± 1.09 l
		2.5	62.61 ± 4.32gh	− 0.86 ± 0.13def	6.67 ± 0.12cdef	0.555 ± 0.03jkl	0.5 ± 0.15efgh	20.16 ± 6.17cde
		5	71.78 ± 5.72hi	− 2.19 ± 0.18a	7.28 ± 0.21efgh	0.408 ± 0.009fghi	1.53 ± 0.36ij	30.63 ± 7.14efg
		15	83.34 ± 1.85j	− 2.04 ± 0.57a	2.58 ± 0.84a	0.205 ± 0.042ab	2.9 ± 0.18 l	19.3 ± 1.19 cd
	25 °C	0	20.73 ± 1.01c	2.27 ± 0.54jk	10.4 ± 0.77i	0.555 ± 0.02jkl	0 ± 0abc	0 ± 0a
		0.625	70.26 ± 2.6hi	− 1.02 ± 0.55cde	4.13 ± 0.46b	0.459 ± 0.029ghij	0.36 ± 0.03de	57.18 ± 5.3k
		2.5	76.3 ± 1.39i	− 1.87 ± 0.45ab	5.62 ± 1.53bcde	0.359 ± 0.045defg	1.02 ± 0.21hi	40.7 ± 8.31hi
		5	54.89 ± 5.08e	− 1.31 ± 0.35bcd	4.94 ± 1.31bcd	0.227 ± 0.046abc	1.35 ± 0.75i	19.04 ± 1.18 cd
		15	71.38 ± 4.79hi	− 0.83 ± 0.38def	4.3 ± 1.77abc	0.275 ± 0.064abcde	0.23 ± 0.16bcde	1.55 ± 1.05a
	18 °C	0	2.76 ± 1.69a	3.18 ± 0.52kl	9.66 ± 1.31ij	0.607 ± 0.024 l	0 ± 0ab	0 ± 0a
		0.625	21.73 ± 3.05c	2.07 ± 0.66jk	9.15 ± 0.27ghij	0.538 ± 0.025jkl	0.43 ± 0.13ef	81.26 ± 1.13 m
		2.5	58.79 ± 0.74ef	0.68 ± 0.22ghij	4.5 ± 1.09abc	0.458 ± 0.059ghij	0.86 ± 0.1fghi	34.55 ± 4.08fgh
		5	76.96 ± 6.04i	− 0.48 ± 0.48defgh	4.53 ± 0.54abc	0.338 ± 0.034cdef	1.13 ± 0.41i	22.56 ± 8.11cde
		15	89.61 ± 1.1k	− 1.11 ± 0.36cde	4.35 ± 0.6abc	0.262 ± 0.038abcd	1.04 ± 0.1hi	6.96 ± 0.67ab
S35	28 °C	0	1.05 ± 0.2a	0.35 ± 0.12fghi	9.93 ± 0.56i	0.504 ± 0.02ijkl	0 ± 0abc	0 ± 0a
		0.625	1.35 ± 0.55a	− 0.41 ± 0.29defgh	8.14 ± 0.89fghij	0.529 ± 0.033ijkl	0.31 ± 0.03cde	50.38 ± 4.29jk
		2.5	2.15 ± 0.64a	− 0.1 ± 0.49efghi	8.24 ± 1.14fghij	0.381 ± 0.06efgh	0.99 ± 0.1hi	39.68 ± 4.03ghi
		5	9.69 ± 2.91b	− 1.25 ± 0.69bcd	6.43 ± 0.34bcdef	0.479 ± 0.024hijk	1.31 ± 0.21i	26.3 ± 4.26def
		15	45.39 ± 5.8d	− 1.75 ± 0.51abc	4.51 ± 0.72abc	0.241 ± 0.05abc	1.96 ± 0.18jk	13.09 ± 1.17 BCE
	25 °C	0	2.67 ± 2.47a	4.99 ± 0.73 lm	12.65 ± 0.82k	0.449 ± 0.042ghij	0 ± 0abc	0 ± 0a
		0.625	2.6 ± 2.28a	3.14 ± 0.78klm	12.31 ± 0.46k	0.507 ± 0.073ijkl	0.5 ± 0.05efg	79.94 ± 7.36 m
		2.5	0.71 ± 0.05a	0.87 ± 0.35hij	9.62 ± 1.32i	0.273 ± 0.031abcde	0.83 ± 0.03fghi	33.18 ± 1.07fgh
		5	0.94 ± 0.14a	− 0.67 ± 0.32defg	7.37 ± 0.64efghj	0.177 ± 0.013a	1.11 ± 0.08i	22.1 ± 1.55cde
		15	9.5 ± 0.52b	− 1.06 ± 0.26cde	4.14 ± 0.36ab	0.183 ± 0.082a	2.37 ± 0.93kl	19.24 ± 1.8 cd
	18 °C	0	0.65 ± 0.17a	5.57 ± 0.45 m	10.02 ± 1.08i	0.616 ± 0.069 l	0 ± 0a	0 ± 0a
		0.625	0.7 ± 0.02a	4.84 ± 0.93 lm	9.07 ± 0.77ghij	0.621 ± 0.02 l	0.27 ± 0.05bcde	43.96 ± 7.87ij
		2.5	0.64 ± 0.1a	0.59 ± 0.11ghij	9.51 ± 1.27hij	0.527 ± 0.037ijkl	0.99 ± 0.46ghi	29.48 ± 6.79def
		5	0.88 ± 0.23a	− 0.6 ± 0.13defg	7.07 ± 0.75defg	0.318 ± 0.098bcdef	1.03 ± 0.11hi	20.65 ± 2.19cde
		15	1.05 ± 0.48a	− 0.32 ± 0.49defgh	5.01 ± 0.27bcd	0.184 ± 0.049a	4.2 ± 0.83 m	27.99 ± 5.57def

Table 2
Summary of Three-Way ANOVA Analysis comparing Bleaching Degree, Growth Rate (GR), Absolute Electron Transport Rate (ETR_{abs}), Maximum Quantum Yield (F_v/F_m), Cadmium Uptake (Cd uptake) and Concentration Factor (CF) of *Ulva ohnoi* exposed to two salinities (15 and 35), three temperatures (18, 25 and 28 °C) and five concentrations of Cd (0, 0.625, 2.5, 5 and 15 μg. L^{-1}) after 8 days. Significant treatments are assigned in bold ($p < 0.05$).

	Bleaching Degree		GR		ETR_{abs}		Fv/Fm		Cd Uptake		CF	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Temperature	87.6	0.000	127.3	0.000	5.9	0.005	45.3	0.000	6.2	0.004	0.1	0.879
[] Cadmium	486.1	0.000	143.2	0.000	107.5	0.000	161.0	0.000	216.9	0.000	577.2	0.000
Salinity	6333.9	0.000	99.5	0.000	132.3	0.000	7.0	0.010	17.0	0.000	0.0	0.893
Temperature* [] Cadmium	51.0	0.000	8.6	0.000	7.5	0.000	8.8	0.000	9.2	0.000	5.3	0.000
Temperature*Salinity	14.2	0.000	9.6	0.000	11.3	0.000	4.3	0.017	9.0	0.000	16.5	0.000
[] Cadmium*Salinity	258.7	0.000	9.5	0.000	6.6	0.000	4.4	0.004	21.9	0.000	16.1	0.000
Temperature* [] Cadmium*Salinity	62.1	**0.000**	7.0	**0.000**	14.8	**0.000**	4.1	**0.001**	13.4	**0.000**	25.6	**0.000**

25 °C, S35, when concentrations were equal to, or greater than, 2.5 μg L^{-1} and treatments of 18 and 28 °C, S35, when the concentration of Cd was equal to, or greater than, 5 μg L^{-1}. The remaining treatments were affected at even lower concentrations (0.625 μg L^{-1}) (Table 1).

The values of Maximum Quantum Yield (F_v/F_m) decreased when Cd concentrations were increased (Table 1). At S15, effects were observed when Cd concentration was equal to, or greater than, 2.5 μg L^{-1} at 18 and 25 °C and 5 μg L^{-1} at 28 °C. However, for S35, the effects were only observed at higher concentrations (2.5 μg. L^{-1} at 25 °C, 5 μg L^{-1} at 18 °C and 15 μg L^{-1} at 28 °C) (Table 1).

The ETR_{abs} x Irradiance PAR_{abs} curves maintained similar shapes in all treatments, but showed different magnitudes between the initial curves and after 4 and 8 days (Fig. 2). All treatments showed a decrease in the absorption of photosynthetically active radiation (PAR_{abs}), especially at higher Cd concentrations (Fig. 2). The amplitude of curves (values of ETR_{abs}) gradually decreased with increasing Cd concentrations and over time in relation to the initial curve. At 18 °C (both salinities), a smaller difference in amplitude was noted between 0.625 μg L^{-1} Cd and time in relation to the initial curve (Fig. 2).

3.5. Cadmium uptake

The Cd contents found in *U. ohnoi* biomass increased with the concentrations used ($r = 0.648$; $p < 0.0001$ level, $N = 72$) and were significantly affected by the treatments used (Tables 1 and 2). Cd accumulation was higher in S35 with a maximum value of 4.199 ± 0.835 μg g^{-1} Cd at 18 °C with 15 μg L^{-1} Cd in water. For S15, the highest accumulation was at 28 °C and 15 μg. L^{-1} Cd in water (2.896 ± 0.178 μg g^{-1} Cd).

The concentration factor (CF) was also significantly influenced by the treatments applied (Table 2). The CF values decreased with the increase of Cd concentration in the medium ($r = -0.685$, $p < 0.0001$ level, $N = 72$). Also, the average CF value was higher in S15 (69.6 ± 12.1%), followed by S35 (58.1 ± 19.2%). For temperature, the average CF value was higher at 25 °C (68.6 ± 16.1%), followed by 18 °C (62.6 ± 26.4%) and 28 °C (60.3 ± 14.1%). The CF values were higher in the lowest Cd concentrations (0.625 μg L^{-1} Cd - 63.8 ± 15.7%), decreasing when the concentration of Cd in the medium increased (2.5 μg L^{-1} Cd - 33.0 ± 7.5%; 5 μg L^{-1} Cd - 23.5 ± 4.2%, 15 μg L^{-1} Cd - 14.7 ± 9.5%). At 0.625 μg L^{-1} Cd, the highest CF value was found in the combination of 18 °C and S15 (81.3 ± 1.1%), followed by 25 °C and S35 (79.9 ± 7.4%) and 28 °C and S15 (70.3 ± 1.1%). For 2.5 μg L^{-1} Cd, CF presented higher values at 25 °C and S15 (40.7 ± 8.3%), followed by 28 °C and S35 (39.7 ± 4.0%) and 18 °C and S15 (35.5 ± 4.1%). At 5 μg L^{-1} Cd, CF presented higher values at 28 °C and S15 (30.6 ± 7.1%), followed by 28 °C and S35 (26.3 ± 4.3%) and 18 °C and S15 (22.6 ± 8.1%). Finally, at 15 μg. L^{-1} Cd, the highest CF values were at 18 °C and S35 (28.0 ± 5.6%), followed by 28 °C and S15 (19.3 ± 1.2%) and 25 °C and S35 (19.2 ± 1.8%) (Table 1).

3.6. Correlation analysis

Cd content found in biomass correlated significantly and positively with the bleaching degree of thalli and negatively with GR and photosynthetic parameters (F_v/F_m and ETR_{abs}) (Table 3). The degree of bleaching correlated negatively with GR, ETR_{abs} and F_v/F_m, and CF data did not present significant correlation with any parameter analyzed (Table 3).

4. Discussion

In recent decades, the introduction of non-native marine species has been increasing (Batista et al., 2018; Silva et al., 2010). Climate change and human activities are among the main causes of these issues (Boudouresque and Verlaque, 2010). On the other hand, increase in the identification of non-native species may result from the use of molecular tools in the identification of taxa and separation of species that have few, or no, distinct morphological characters (Hayden and Waaland, 2004), e.g., seaweeds of the genus *Ulva* (Guiry and Guiry, 2018).

Some species of the genus *Ulva* have shown dispersion and invasion ability, complicating species identification (Hayden and Waaland, 2004). Based on molecular analyses, we detected the presence of *U. ohnoi*, a possible non-native species occurring along the Brazilian coast, adding this species to the marine flora of the South Atlantic. In the same way, a growing number of *U. ohnoi* records have been reported. This species was initially described by Hiraoka et al. (2004b) from algae found in Tosa Bay, Japan, using morphological and molecular analyses (rbcL and ITS1–5.8S-ITS2), as well as crossing among species (Hiraoka et al., 2004a). After its description, *U. ohnoi* was subsequently recorded in different locations, such as Japan (Kawai et al., 2007; Yabe et al., 2009), Hawaii (O'Kelly et al., 2010), Italy (Flagella et al., 2010), Australia (Kirkendale et al., 2013), USA (Melton et al., 2016a), Venezuela (Melton et al., 2016b) and Tunisia (Miladi et al., 2018).

U. ohnoi has aroused great interest because of its potential for nitrogen and phosphorus absorption from aquaculture effluents (Lawton et al., 2013; Masaló et al., 2016), producing biomass with high levels of proteins, lipids, amino acids and sulphated polysaccharides (Angell et al., 2015, 2014; Glasson et al., 2017; Mata et al., 2016). These characteristics make the species a potential tool for the uptake of metallic ions from contaminated saline waters.

In this work, *U. ohnoi* showed growth in all temperature and salinity treatments used, with highest growth in temperatures of 18 °C and 25 °C and S35. These data partially corroborate the values found by Ohno (1988) (cited as *Ulva* sp.) for the site of the type species with higher GR between 20 and 28 °C and lowest at 18 °C in mean salinity of 18. However, Notoya (1999) found optimal condition between 20 and 25 °C, and Lawton et al. (2013) found different responses of seven strains found in Australia. This species still presents a wide range of growth in different salinities (20–60) with optimum growth between 25

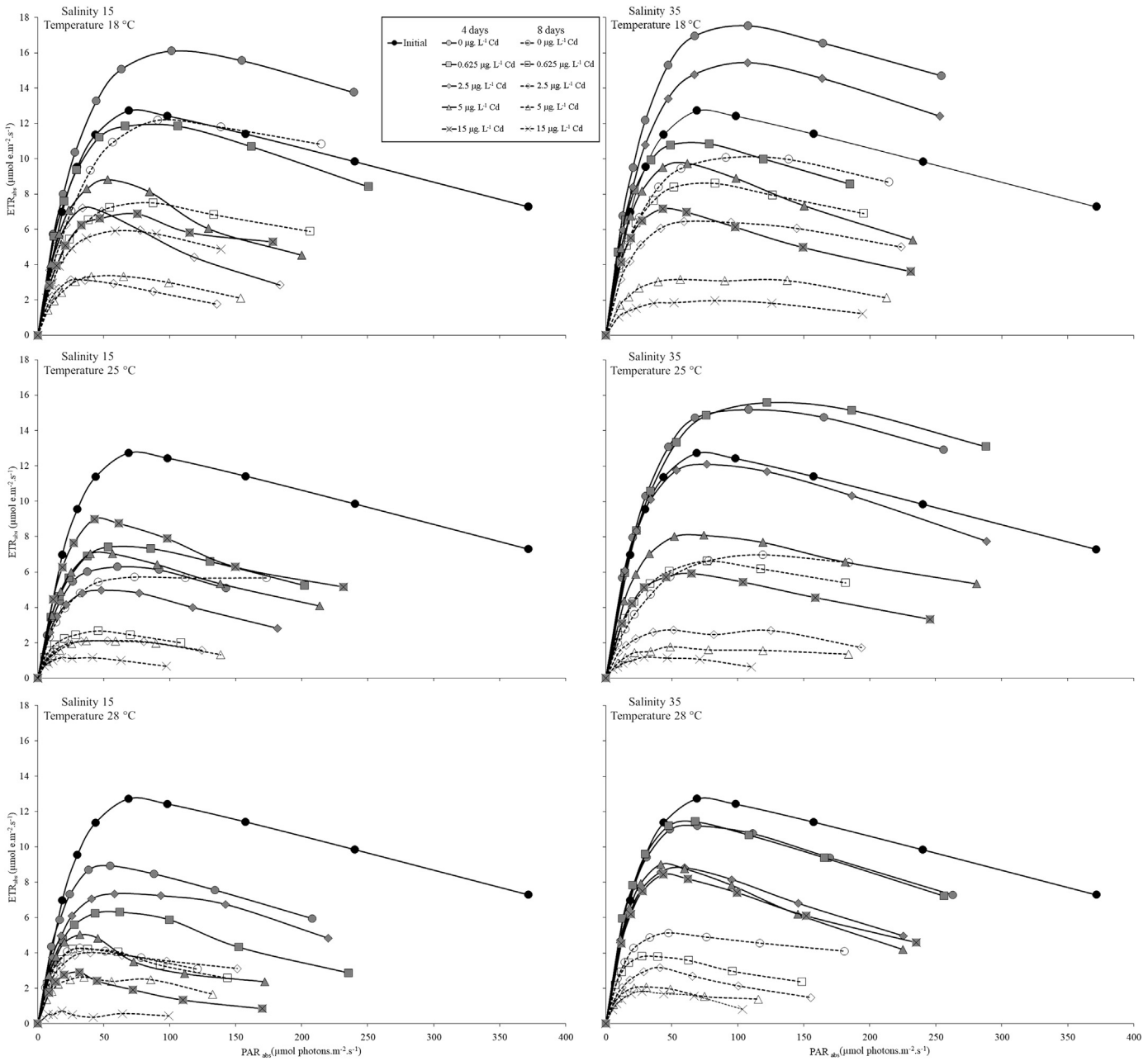

Fig. 2. Absolute Electron Transport Rate (ETR_{abs}) at increasing PAR_{abs} intensities (ETR_{abs} x Irradiance PAR_{abs} curves) of *Ulva ohnoi* exposed to two salinities (15 and 35), three temperatures (18, 25 and 28 °C) and five concentrations of Cd (0, 0.625, 2.5, 5 and 15 µg. L^{-1}) after 4 and 8 days. Points represent mean for N = 3.

Table 3
Pearson correlation values obtained among dependent variables after treatment of *Ulva ohnoi* exposed to two salinities (15 and 35), three temperatures (18, 25 and 28 °C) and five concentrations of Cd (0, 0.625, 2.5, 5 and 15 µg L^{-1}) after 8 days. Significantly correlated treatments are assigned in bold ($p < 0.05$). Dependent variables: Bleaching Degree, Growth Rate (GR), Absolute Electron Transport Rate (ETR_{abs}), Maximum Quantum Yield (F_V/F_m), Cadmium Uptake (Cd uptake) and Concentration Factor (CF).

	GR	ETR_{abs}	Fv/Fm	Cd Uptake	CF
Bleaching Degree	**− 0.571**	**− 0.618**	**− 0.261**	**0.232**	0.037
GR		**0.675**	**0.560**	**− 0.600**	− 0.087
ETR_{abs}			**0.551**	**− 0.572**	0.127
Fv/Fm				**− 0.741**	0.134
Cd Uptake					0.140

and 40 (Angell et al., 2015). This heterogeneity of responses to temperature and salinity of *U. ohnoi* can be attributed to genotypic adaptations to local conditions (Eggert, 2012; Karsten, 2012), as well as the phenotypic plasticity of the species, which explains the diversity of new sites and environmental conditions where the species is being reported (Guiry and Guiry, 2018). These eurythermal and euryhaline characteristics are very important for phytoremediation since the conditions of effluents or impacted environments are diverse (Lawton et al., 2013).

Although *U. ohnoi* found in Brazil grows in different environmental conditions, GRs reported here (see Table 1) are low compared to those found for this species in other localities (Angell et al., 2014, 2015; Lawton et al., 2013). We observed a decrease of GRs at 28 °C (both salinities), an increase in bleaching at S15 and a reduction in PAR_{abs} and amplitude of ETR_{abs} x Irradiance PAR_{abs} curves over time (0, 4 and 8 days) in all treatments. It is possible that this response results from the amount of nitrogen (0.5 mM) added to the medium during the

experiment. It is well known that the availability of nitrogen in coastal environments of temperate regions is considered the main limiting factor for macroalgae growth, and when found in excess, it has stimulated uncontrolled growth of *U. ohnoi* in some sites (Howarth et al., 2000; Melton et al., 2016b; Nixon and Pilson, 1983). When nitrogen becomes limiting, the pigments are metabolized, generating thalli bleaching (see Table 1) to obtain nitrogen and maintain osmotic regulation and other metabolic processes (Floreto et al., 1993; Gómez Pinchetti et al., 1998). Although low GRs were found here, the results obtained do not invalidate the use of *U. ohnoi* for phytoremediation of Cd, but they do demonstrate the need for better studies with the Brazilian strain where the supply of nitrogen and phosphorus are experimentally varied. Angell and et al. (2014, 2015) demonstrated the growth potential of the species when nitrogen concentrations increased in the water, obtaining GR of 15.04% day^{-1} to 26.8% day^{-1}.

When Cd was added, we observed a dose-dependent effect with higher toxicity when *U. ohnoi* was exposed to higher concentrations. This effect was also observed by Kumar et al. (2010) for *U. lactuca* and by Jiang et al. (2013) for *U. linza* Linnaeus and *U. prolifera* O. F. Müller. Under the influence of temperature and salinity, we observed that metal toxicity was higher with lower salinity and higher with higher temperature, reducing the photosynthetic parameters analyzed, consequently, the GR values. A similar pattern was found by Connan and Stengel (2011) who reported that an increase in copper concentrations and reduced salinity physiologically affected the brown seaweed *Ascophyllum nodosum* (Linnaeus) Le Jolis. Although temperature is very important for macroalgae, research on its interaction with metal toxicity has been neglected. On the other hand, studies with microalgae are common and show increased toxicity with increasing temperature (Rai et al., 1981).

In this work, *U. ohnoi* maintained its growth and high photosynthetic activity when exposed to the lowest concentrations of Cd (0.625 and 2.5 μg. L^{-1}) at S15 and 18 °C and at S35 and both 18 and 25 °C. Resistance to low concentrations was also reported by Jiang et al. (2013) for *U. prolifera* and *U. linza*. The authors observed an increase in the capacity of osmotic control and maintenance of total N and P contents at low concentrations of Cd. This ability has also been reported in halophytes that exhibit resistance to Cd, but their resistance mechanisms remain unclear (Ghnaya et al., 2007). Negative GRs with the lowest concentration of Cd (0.625 and 2.5 μg L^{-1}) were observed in the treatments that showed lower GRs without addition of Cd (S15, 25–28 °C e S35, 28 °C). These reductions in GRs may be related to mechanisms of exclusion or immobilization of the metal by active processes aimed at reducing toxicity. With part of the metabolism reallocating energy to reduce the stress of temperature and salinity, the addition of Cd may have resulted in a negative energy balance. Similar results with the interaction of salinity and trace metal were also observed by Connan and Stengel (2011) for *Ascophyllum nodosum* and *Fucus vesiculosus* Linnaeus with reduction of GRs at the lowest copper concentration (0.1 mg L^{-1} Cu) and negative values from 1 mg L^{-1} Cu.

The performance of photosynthesis, as indicated by ETR_{abs}, F_v/F_m and ETR_{abs} x Irradiance PAR_{abs} curves, showed a significant reduction with increasing concentration of Cd in the medium and a high correlation with Cd uptake (see Table 3). Several patterns were found in the salinities and temperatures used, demonstrating the complexity of interactions that occur in trifactorial experimentation. Our results indicated that Cd concentration influenced light absorption by photosystem II in *U. ohnoi*. The PAR_{abs} values (see Fig. 2) demonstrated reduction over time and dose-dependent characteristics, resulting in a reduction of ETR_{abs} values. This process results from the multiple binding sites of Cd in the components of photosynthetic processes (Kučera et al., 2008). The components of the primary photochemistry of photosystem II are more susceptible to Cd interference with both the donor side and the electron receiving side in the electron transport rate (Perreault et al., 2011). In the donor, the Ca exchange for Cd occurs in the Mn cofactor affecting the Hill reaction and inhibiting oxygen evolution (Parmar et al., 2013; Perreault et al., 2011). In the receptor, Cd causes several effects, such as interference in the electron transfer between plastoquinones QA and QB (Parmar et al., 2013; Sigfridsson et al., 2004). It should also be noted that Cd replaces Mg in the center of the chlorophyll molecule, disabling photon absorption and transferring to the reaction centers, resulting in chlorophyll degeneration (Bertrand and Poirier, 2005). This fact can also explain our results of thallus bleaching caused by pigmentation loss in treatments where Cd was added.

Although Cd causes physiological changes compromising physiological processes, in this work, *U. ohnoi* presented growth maintenance, even when grown in water contaminated with Cd (see Table 1). We also observed a dose-dependent accumulation of Cd in all combinations used with a maximum value of 4.20 μg g^{-1} Cd (see Table 1). Dose-dependent accumulation is attributed to increased driving force caused by the difference in Cd concentration between medium and algae (Suzuki et al., 2005), as well as the binding sites available on the cell wall and intracellular material (Webster et al., 1997). The maintenance of growth may be related to a greater amount of Cd binding sites in the cell wall, making it difficult to internalize, resulting in metabolic damage.

Maximum concentration factor (CF) values were 81.3 ± 1.1% of the Cd added at 0.625 μg L^{-1} at S15 and 18 °C, followed by 79.9 ± 7.4% at S35 and 25 °C. The CF values decreased with increasing Cd concentration in the medium, with low values (13.1 ± 1.2% for S35, 28 °C and 1.5 ± 1.1% for S15, 25 °C) at the maximum concentration used (15 μg L^{-1}). In fact, live *U. ohnoi* exhibits greater efficiency in Cd uptake than other live macroalgae (see Table 4). This difference in uptake efficiency may be linked to initial Cd concentrations used in the studies. It is noteworthy that the values of Cd used in the present study represent a gradient around the values permitted by Brazilian legislation for marine and estuarine waters (CONAMA, 2005). Thus, our findings may represent, in a sense, real world scenarios. In contrast most of studies evaluating the toxicity and biochemical effects of Cd contamination on macroalgae use extremely high concentrations compared to those found in effluents or waters contaminated by effluent discharges (Ferreira et al., 2004). Even if they have high values of uptake efficiency (see Table 4), the final levels in

Table 4
Recent reports on metal uptake efficiency of macroalgae.

Macroalgae	Initial concentration of Cd	Metal uptake efficiency (%)	Exposure time	References
Ulva lactuca	1.50 mg L^{-1}	27.04	5d	Saleh (2015)
Ulva prolifera	1.12 mg L^{-1}	39.2	7d	Jiang et al. (2013)
Ulva linza	1.12 mg L^{-1}	24.3	7d	Jiang et al. (2013)
Ulva intestinalis	1.12 mg L^{-1}	12.5	7d	Vecchia et al. (2012)
Ulva laetevirens	1.12 mg L^{-1}	30.7	7d	Vecchia et al. (2012)
Ulva ohnoi	**0.625 μg L^{-1}**	**81.3**	**8d**	**This work**
Pterocladiella capillacea	0.17 mg L^{-1}	75.6	7 d	Felix et al. (2014)
Gracilaria tenuistipitata	1 mg L^{-1}	9.1	6d	Tonon et al. (2011)
Fucus vesiculosus	10 μg L^{-1}	76.3	7d	Henriques et al. (2017)

the solution found in these studies are still higher that those permitted by Brazilian legislation, making potential bioremediation less relevant in such studies.

Our results demonstrated that live *U. ohnoi* has acceptable Cd uptake capacity over a range of combinations of temperature, salinity and realistic concentrations of Cd. At the lower concentrations used, the seaweed maintained its growth (cell multiplication), resulting in increased surface area and possibly increased number of available binding sites for Cd. However, the results from treatments without Cd addition demonstrated that *U. ohnoi* may have been limited by nutrients in the last 4 days of the experiment (see Fig. 2). Nitrogen deficiency may lead to a decrease in enzymatic activity of carbon metabolism and a decline in the concentration of soluble proteins (Pinchetti et al., 1998). Thus, the uptake efficiency of Cd by *U. ohnoi* may be higher than herein recorded. Studies that link the uptake of Cd and nutrients in *U. ohnoi* are needed to better elucidate this issue. This assumption was confirmed for *U. fasciata* for which Lee and Wang (2001) obtained a 2.4 times increase in Cd accumulation when nitrate concentration in the medium was increased from 10 to 100 μM.

5. Conclusion

This work demonstrated the phytoremediation potential of *U. ohnoi* under environmentally realistic conditions of Cd contamination. In the same way, the effects of physicochemical parameters (salinity and temperature) on toxicity and trace metal uptake capacity were evaluated, elucidating, as far as possible, the complex relationships among the factors used and the biological responses of this species. In general, changes in the photosynthetic parameters analyzed were observed at all Cd concentrations used. However, in some combinations of salinity, temperature and Cd concentration, these changes were not reflected in growth reduction and increase in thallus bleaching. This fact occurred in the same combinations that favored growth in treatments without Cd (18 °C, S15 and 18–25 °C, S35), indicating that under these conditions, the negative effects of Cd are not sufficient to negatively alter the metabolic balance. These conditions allowed the growth and continued metal absorption from the medium, lowering residual Cd concentrations in seawater. Dose-dependent accumulation was observed in all factors and combinations used with a maximum value of 4.20 μg Cd per gram of dry seaweed when *U. ohnoi* was exposed to 15 μg L^{-1} of Cd at 18 °C and S35. The maximum CF values were 81.3 ± 1.1% of the Cd added at the concentration of 0.625 μg L^{-1} at S15 and 18 °C, followed by 79.9 ± 7.4% at S35 and 25 °C.

In general, our results demonstrated the potential of using *U. ohnoi* in the phytoremediation of Cd in contaminated waters with the added advantage of having high nutrient absorption rates and high growth rates. Thus, it may be a promising species for new studies aimed at a phytoremediation model for seawater contaminated by other trace metals.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2019.01.130.

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