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Prospects in Cadmium-Contaminated Water Management Using Free-Living Cyanobacteria (*Oscillatoria* sp.)

Iván Carralero Bon [1,2], Lucas M. Salvatierra [1,2], Luciana D. Lario [1,2], Jordi Morató [3] and Leonardo M. Pérez [1,2,3,*]

1 Grupo de Biotecnología de Materiales y Medioambiente (Bio&TecMA), Instituto de Investigaciones en Ingeniería Ambiental, Química y Biotecnología Aplicada (INGEBIO-UCA), Facultad de Química e Ingeniería del Rosario, Pontificia Universidad Católica Argentina (UCA), Av. Pellegrini 3314, Rosario S2002QEO, Argentina; icbon89@gmail.com (I.C.B.); lucas_salvatierra@uca.edu.ar (L.M.S.); lario@rosario-conicet.gov.ar (L.D.L.)

2 National Council for Scientific and Technical Research (CONICET), Ministry of Science, Technology and Productive Innovation, Godoy Cruz 2290, Buenos Aires C1425FQB, Argentina

3 UNESCO Chair on Sustainability, Universitat Politècnica de Catalunya-BarcelonaTech, C/Colom, 1, TR1, ESEIAAT, 08222 Terrassa, Spain; jordi.morato@upc.edu

* Correspondence: perez@inv.rosario-conicet.gov.ar; Tel.: +549-341-421-130

Abstract: In this study, the removal of cadmium (Cd) by free-living *Oscillatoria* sp. was studied. Our results showed that maximal Cd removal efficiency (~60%) by the cyanobacterial culture was achieved within 12–24 h in the presence of 5.0 or 25.0 mg/L of Cd. The mechanisms underlying this phenomenon were explored by elemental analysis and FTIR-ATR spectroscopy. It was found that metal adsorption by negatively charged functional groups in the cyanobacterial biomass was the main mechanism used by *Oscillatoria* sp. to remove metal from the aqueous medium, followed by Cd bioaccumulation into living cells. Additionally, Cd-exposed microalgae showed increased oxidative stress (MDA formation), a decreased dehydrogenase activity, a higher amount of soluble carbohydrates and a decreased total carotenoid concentration, as compared to the control cells. These results suggest that *Oscillatoria* sp. improved its antioxidative defense system under stressful conditions, through carotenoid-mediated ROS quenching and induction of carbohydrate catabolism, in order to counteract the oxidative damage and preserve the photosynthetic machinery and cellular energetics. In fact, no significant reduction in *Oscillatoria* sp. cell density, total protein amount, and chlorophyll a content was observed after 24-h Cd exposure, even at the highest metal concentration tested (i.e., 25.0 mg/L). Hence, the presented results are the first to describe some new insights about the metabolic and physiological behavior of living *Oscillatoria* sp. during Cd remediation, and open up the possibility of finding an equilibrium that maximizes metal removal performance with an active cyanobacterial metabolism, to achieve a rewarding and sustainable management of industrial metal-polluted wastewater.

Keywords: cadmium-polluted water; ecotechnologies; bioremediation; free-living cyanobacteria; *Oscillatoria* sp.

1. Introduction

Heavy metal pollution of fresh-water reservoirs is one of the most relevant environmental issues since toxic metal persistence in water poses a serious risk to both aquatic organisms and human beings [1]. Most metals are waste products of industrial and metallurgical processes and their environmental impact is largely increased due to the continuous release of aqueous effluents containing heavy metals without an appropriate treatment. This malpractice is somewhat frequent in poor and developing countries where sanitary solutions are weak and scarce, and toxic metals are often discharged into natural water sources without adequate handling, and poses a major health risk [2,3].

In particular, cadmium (Cd) is one of the most toxic metals often linked to human poisoning. Common sources of Cd include wastes from Cd-based batteries and runoff water from agricultural soils impacted with Cd-containing phosphate fertilizers. In addition, a large amount of Cd is naturally released into rivers through the weathering of rocks. Cadmium is also used in mining, metallurgical operations, electroplating industries, manufacturing of vinyl plastics, and as a pigment in paint production [4]. Furthermore, Cd is present in most foodstuffs, such as cereal products, vegetables, nuts, starchy roots, and meat products [5]. Therefore, Cd exposure is still a global concern.

Although Cd toxicity is a highly-explored matter, complete control over Cd pollution is still far from being achieved and cases of Cd poisoning are still reported. Long-term human exposure to high levels of Cd induces renal damage associated with kidney and chronic liver dysfunction. In addition, this element is classified as a Group 1 human carcinogen, based on data reporting an increased risk of prostate, lung, endometrium, bladder, and breast cancer, related to Cd exposure [4,6]. Moreover, Cd is found to be highly toxic to fish and other aquatic organisms [7,8]. Thus, Cd is considered a priority water pollutant from a monitoring perspective by most countries and international organizations. In fact, this element is currently listed by the World Health Organization (WHO) as one of the chemicals of major public concern [9]. According to WHO's recommendation, the Cd limit in drinking water is 0.005 mg/L. Therefore, the treatment or purification of Cd-polluted water and effluents is an active research area.

Several physicochemical technologies such as chemical flocculation/precipitation, ion exchange, solvent extraction, reverse osmosis, electrochemical deposition, and membrane filtration are usually employed to clean up metal-polluted waters and remove heavy metals from industrial effluents [10,11]. However, many of these solutions are technically infeasible, expensive, environmentally hazardous, or far away from the desired performance [12]. In the last decades, the search for cost-effective biotechnologies for the eco-management of metal-containing wastewaters gained a lot of attention [13,14]. Besides its eco-friendly spirit, these new biotechniques should be efficient enough to comply with the current legislation demands [12]. In this context, research efforts were made for Cd removal from aqueous systems based on biosorption techniques. Various biomaterials such as bacteria, fungi, yeasts, algae, and plants were employed to investigate effective Cd-biosorption systems [15–18].

Cyanobacteria are also excellent models for metal bioaccumulation studies. These organisms are the largest and most diverse group of photosynthetic prokaryotes that have the ability to survive in both fresh and marine brackish waters. During evolution, cyanobacteria developed diverse strategies to maintain an equilibrated relation with heavy metals in the surrounding medium [19]. Therefore, these microorganisms are excellent candidates for an efficient elimination of metal ions from polluted environments [20], especially those cyanobacteria that lack commercial value (e.g., for food purposes).

Heavy metal toxicity against cyanobacteria depends on the metal type and concentration, the taxon (i.e., the types of cyanobacteria), and the environmental conditions such as temperature, pH, and light conditions [7,13,14,19,20]. Living cells are likely to be more sensitive to metal ions. Therefore, most studies oriented to heavy metals removal from aqueous systems are made using dried, non-living, or pre-treated cyanobacterial biomass [15,16,20,21]. However, metabolically-active cyanobacteria can also be used for large-scale production of energy, biofertilizers, secondary metabolites, cosmetics, and medicines [22–24]. Therefore, in removing metal ions from wastewaters, living cyanobacteria could be used as an eco-friendly sustainable practice coupled with biomass production. This entails the additional benefits of increasing the oxygen supply to the treatment system, with the avoidance of anoxic zones, decreasing the CO_2 levels, or converting radiant energy into chemical energy for further industrial usage [23,25,26].

In the present study, a non-market, self-flocculating, and cost-effective filamentous cyanobacterium (*Oscillatoria* sp.) was investigated for Cd elimination from artificially-contaminated water solutions. The effect of metal concentration on oxidative damage and

the Cd removal capacity of living cells was explored. In addition, the different uptake mechanisms used for *Oscillatoria* sp. during Cd removal were also assessed. Finally, the effects of Cd bioaccumulation on cyanobacterial cell viability, photosynthetic pigment content (i.e., chlorophylls and carotenoids), and carbohydrate and protein biosynthesis were also evaluated.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

Oscillatoria sp. inoculum was kindly provided by a local producer, through the National Institute of Agricultural Technology (INTA-Rosario, Centro Regional Santa Fe, Argentina). Taxonomic classification was carried out by the *Instituto del Alimento* (Secretaría de Salud Pública, Municipalidad de Rosario, Argentina), based on the morphological characteristics. Pure microalgae culture (>90%) was grown in a modified Zarrouk's medium (pH 7.4) containing $NaHCO_3$ (16.8 g/L), K_2HPO_4 (0.5 g/L), $NaNO_3$ (2.5 g/L), K_2SO_4 (1.0 g/L), NaCl (1.0 g/L), $MgSO_4$ (0.2 g/L), $CaCl_2$ (0.04 g/L), and 1 mL of a solution consisting of H_3BO_3 (2.86 g/L), $MnCl_2$ (1.81 g/L), $CuSO_4$ (0.222 g/L), and MoO_3 (0.015 g/L). The cells were incubated at 24 ± 2 °C in a glass aquarium adapted with air diffusers, and under a cool white fluorescent light intensity of 100 µE m^{-2} s^{-1} (Osram Dulux L HE, Munich, Germany) in 12-h light–dark cycles, which is suitable for photosynthesis. Before the experiments, the biomass was harvested by centrifugation at 4000 rpm for 10 min, using a Tyfon II centrifuge (Zelian S.A., Buenos Aires, Argentina). The cell pellets were washed twice with saline solution (NaCl 0.9 g/L) to eliminate trace elements that might potentially interfere with the metal binding properties. At the end of the experimental period (24 h), the cyanobacterial biomass was treated for further analytical analysis. In order to determine the dry weight of the biomass, 50.00 mL of culture samples (n = 2) were filtered using previously tared filter papers (Whatman™ Grade 589/3, Maidstone, UK) and oven-dried to a constant weight at 80 °C for 24 h. An average initial cyanobacterial biomass concentration of 0.58 ± 0.07 g (dry weight) was used in all experiments.

2.2. Stock Solution of Cd

Cadmium (Cd) stock solution (10,000 mg/L) was prepared by dissolving analytical grade $CdCl_2$ (Cicarelli, San Lorenzo, Argentina) in deionized water. The pH of the stock solution was adjusted to 6.0 ± 0.2 with 1 N HNO_3 (Cicarelli, San Lorenzo, Argentina), using an AD1030 digital pH-meter (Adwa, Nușfalău, Romania), in order to avoid $Cd(OH)_2$ precipitation, and then this solution was stored at 4 °C. For calibration purposes, standard solutions of Cd (0.30, 0.60, 1.0, 2.0, 2.5, and 3.0 mg/L) were prepared by diluting the stock solution with the necessary volume of acidified water (0.15% *v*/*v* HNO_3; Cicarelli, San Lorenzo, Argentina) in glass calibrated containers, as recommended by the Varian AA240FS (Varian Inc., Palo Alto, CA, USA) operational manual. All calibration procedures showed reproducible linear relationships (R^2 > 0.98).

2.3. Cd Removal Studies and Elemental Analysis

The removal of Cd by *Oscillatoria* sp. was examined by incubating the cells on phosphate buffer (pH 7.4) supplemented with 0.0 (control), 5.0, or 25.0 mgCd/L. Experiments were performed in duplicates with 250 mL Erlenmeyer flasks containing 150 mL culture, and were incubated at 24 ± 2 °C with dark/light cycles of 12 h, under gentle orbital agitation (80 rpm), to avoid cell sedimentation. Culture samples (10.00 mL) were withdrawn after 0, 4, 8, 12, and 24 h, and the biomass was harvested by centrifugation (4000 rpm, 10 min). The supernatant was then filtered through a PTFE filter (pore size = 0.45 µm), and residual Cd concentration was measured by atomic absorption, using a Varian AA240FS spectrophotometer (Varian Inc., Palo Alto, CA, USA). Additionally, the pellet was treated for elemental analysis. In brief, the cyanobacterial biomass was washed with 10.00 mL of 1.7 mM EDTA solution for 60 min in an orbital shaker, at 180 rpm. Samples from this washing were filtered (pore size = 0.45 µm) and used to quantify the Cd adsorbed on the cyanobacterial

surface through atomic absorption spectrophotometry [27]. The EDTA-washed biomass was then rinsed with deionized water and treated with 1.0 mL 65% HNO_3 analytical grade (Cicarelli, San Lorenzo, Argentina). Subsequently, the samples were heated at 70 °C in a digestion system, for 3 h. Finally, the samples were diluted with water up to a final volume of 10.00 mL, and then filtered with a pore size of 0.45 μm; the Cd accumulated in cells was spectrophotometrically quantified. Data were reported as the mean value ± standard deviation (S.D.) of three replicates ($n = 3$).

2.4. Metal Removal Kinetics

Kinetic models are frequently used to describe the reaction order of pollutant elimination in bio-based wastewater treatment systems, since the rates of contaminant removal can provide important information concerning the mechanism and behavior at which biological interactions occur and can be used to guide the system design [28]. In general, the dynamics of such bioprocesses can be properly described by the first-order (1) or second-order (2) reaction models, by applying the following equations:

$$\ln C_t = \ln C_0 - K_1\, t \tag{1}$$

$$\frac{1}{C_t} = \frac{1}{C_0} + K_2 t \tag{2}$$

In this study, C_t is Cd residual concentration (mg/L) at time t (h), C_0 is the initial metal concentration in the sample medium (5.0 mg/L or 25.0 mg/L), K_1 represents the first-order reaction rate constant, and K_2 describes the second-order reaction rate constant [29].

2.5. FTIR-ATR Analysis

The spectra of dried *Oscillatoria* sp. biomass were obtained using an IR Prestige-21 Fourier Transform Infrared (FTIR) spectrophotometer (Shimadzu, Tokyo, Japan) in the Attenuated Total Reflectance (ATR) mode, to examine active groups on the cyanobacterial biomass with/without Cd (25.0 mg/L) exposure for 24 h. A total of 20 scans over a recording range of 500–4000 cm^{-1} were performed at 2 cm^{-1} resolution [28].

2.6. Analytical Analysis

2.6.1. Microbial Growth

The cyanobacterial growth after 24 h of Cd exposure at the different metal concentrations tested (i.e., 0.0, 5.0, or 25.0 mg/L) was assessed turbidimetrically as the optical density of the cultures at 560 nm (O.D.$_{560}$) [30], using a UV-Vis Lambda 25 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). The experiments were performed in triplicates ($n = 3$).

2.6.2. Protein Content

The protein content was determined as described by [31]. In brief, two-milliliter (2.0 mL) aliquots of culture samples were sonicated for 30 min in an ultrasonic TB-04 TA water bath (TestLab, Buenos Aires, Argentina). Then, a 0.4 mL portion of this solution was placed in a test tube and 2.0 mL of a solution containing 2% (*w/v*) Na_2CO_3 diluted in 0.1 M NaOH, 1% (*w/v*) $CuSO_4.5H_2O$, and 2% (*w/v*) potassium–sodium tartrate (in proportion: 50:0.5:0.5) were added. The resulting mixture was vortexed for 30 s and incubated in dark for 10 min at room temperature. Finally, 0.2 mL of the Folin-Ciocalteau reagent were added to the solution and vortexed for another 30 s. After a 30 min incubation at room temperature, the absorbance of the sample was measured at 750 nm, using a UV-Vis Lambda 25 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). Absorbance values were converted to protein concentration (mg/L) using a calibration curve established with bovine serum albumin (BSA; Sigma-Aldrich, Steinheim, Germany). Data were reported as the mean value ± standard deviation (S.D.) of three replicates ($n = 3$).

2.6.3. Lipid Peroxidation (MDA Accumulation)

Lipid peroxidation was evaluated based on the TBARS (i.e., thiobarbituric acid reactive substances) determination, using a modification of the method described by [32]. In brief, cyanobacterial cells (1.0 mL culture sample) were harvested by centrifugation at 4000 rpm for 10 min, the supernatant was discarded, and the remaining biomass was homogenized with 1.0 mL of 0.5% (*w/v*) thiobarbituric acid solution in 20% (*w/v*) trichloroacetic acid, added with 0.1 mL of 4% (*w/v*) BHT. The homogenate was boiled for 45 min at 100 °C and centrifuged at 10,000 rpm for 10 min to collect the supernatant. After being cooled at 4 °C, the absorbance of the resulting solution was recorded at 530 nm, using a UV-Vis Lambda 25 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA), and corrected for unspecific turbidity by subtracting the value at 600 nm. Malondialdehyde (MDA) concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as μmol MDA/mg protein. Data were reported as the mean value ± standard deviation (S.D.) of three replicates ($n = 3$).

2.6.4. Dehydrogenase Activity

A decrease in the number of metabolically active cells correlated with a decrease in overall activity of the dehydrogenases responsible for conversion of 2,3,5-triphenyltetrazolium chloride (TTC) to red insoluble 1,3,5-triphenylformazan, a compound that could be spectrophotometrically determined at 485 nm [33]. Therefore, this colorimetric assay was used to evaluate cyanobacterial viability after Cd exposure. In brief, 10.00 mL culture samples treated for 24 h with 0.0 (control), 5.0, or 25.0 mgCd/L were loaded with 0.5 mg/mL TTC, and incubated for 1 h (37 °C, 130 rpm) in a shaking bath (SHZ-88, Arcano, Shanghai, China). Then, cells were harvested by centrifugation (4000 rpm, 10 min) and the product of the hydrolytic reaction (i.e., formazan) was extracted in 1.0 mL of 80% acetone. After centrifugation (10,000 rpm, 10 min), formazan was determined by measuring the absorbance of the supernatant at 485 nm, using a UV-Vis Lambda 25 spectrophotometer (Perkin Elmer, Inc., Waltham, MA, USA). The experiments were performed in triplicate ($n = 3$).

2.6.5. Physiological Parameters
Quantification of Photosynthetic Pigments

Chlorophyll-*a* (*chl-a*) and total carotenoids were obtained by methanolic extraction and quantified by UV-Vis spectrophotometry according to the methods described in [34]. In brief, a 2.0 mL culture was collected by centrifugation at 4000 rpm for 10 min. After discarding the supernatant, 2.0 mL of absolute methanol (Merck, Darmstadt, Germany) were added to the cells, followed by vortex agitation. The sample was then kept in the dark at 4 °C for 24 h, before centrifugation (10,000 rpm, 10 min). The absorbance of the supernatant was measured at 470 nm and 665 nm. The contents of *chl-a* and total carotenoids were estimated by the following equations:

$$chl\text{-}a \text{ (μg/mL)} = 13.43 \text{ (}A_{665}\text{) } v/b \text{ V}$$

$$carotenoids \text{ (μg/mL)} = 4.4 \text{ (}A_{470}\text{) } 10)/b \text{ V}$$

where A_{665} and A_{470} are the absorbance values at 665 nm and 470 nm, respectively, against a blank of the solvent used (i.e., methanol); *v* means the volume of solvent used (mL), *b* is the spectrophotometric cell length (1 cm), and V is the sample volume (mL). The concentrations of the photosynthetic pigments were expressed as μg/mg protein. Data were reported as the mean value ± standard deviation (S.D.) of three replicates ($n = 3$).

2.6.6. Carbohydrate Determination

Carbohydrates were quantified according to the method described by [35]. In short, 2.0 mL of each Cd-treated sample at the end of the assay (24 h) were taken in separate test tubes, and the cells were pelleted at 4000 rpm for 10 min. The cell biomass was then mixed with 2.0 mL of 80% (*v/v*) methanol and heated at 70 °C for 30 min. After being

cooled, 1.0 mL of the extract was mixed with 1.0 mL of 5% (*v/v*) phenol (Sigma-Aldrich, St. Louis, MO, USA), and 5.0 mL of 95% (*v/v*) H_2SO_4 (Cicarelli, San Lorenzo, Argentina). Later, the mixtures were incubated for 1 h at room temperature and the absorbance of the supernatant was measured at 640 nm, using a UV-Vis Lambda 25 spectrophotometer (Perkin Elmer Inc., Waltham, MA, USA). Finally, absorbance values were converted to carbohydrate concentration (mg/L) using glucose (50 mg/mL; Sigma-Aldrich, St. Louis, MO, USA) as a standard [27]. Carbohydrate concentration was expressed as µg/mg protein. Data were reported as the mean value ± standard deviation (S.D.) of three replicates (*n* = 3).

2.7. Statistical Analysis

Statistical analyses were performed using the SigmaStat 3.5 program (Systat Software Inc., San Jose, CA, USA). The ANOVA test was used to compare data between groups (i.e., control and Cd-treated samples). Tukey's honest significant difference (HSD) *post-hoc* test was applied when the differences in the measured values between groups were different ($p < 0.05$).

3. Results

3.1. Cd Removal Efficiency

The capacity of free-living *Oscillatoria* sp. to remove Cd from artificially-contaminated aqueous samples is shown in Figure 1. As can be appraised, Cd concentration from the medium (PBS, pH 7.4) decreased over time, and most of this reduction took place during the first 12–24 h, at both tested metal concentrations (i.e., 5.0 and 25.0 mg/mL). In addition, overall Cd removal performance by *Oscillatoria* sp. was negatively affected by the metal concentration increase. A maximal removal rate of 66.0% was reached when the cyanobacterial cells were exposed to 5.0 mg/L of Cd for 24 h. However, at a metal concentration 5 times higher (i.e., 25.0 mg/L), a slight decrease of around 7% (58.8%) was recorded in Cd removal from the aqueous system at the same period of time. These results suggest that *Oscillatoria* sp. might pose a series of biological mechanisms to deal with Cd intracellular toxicity.
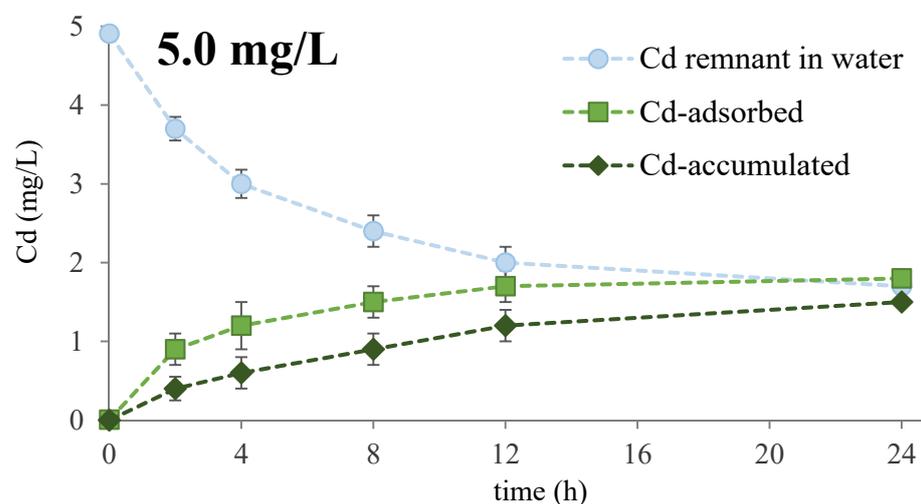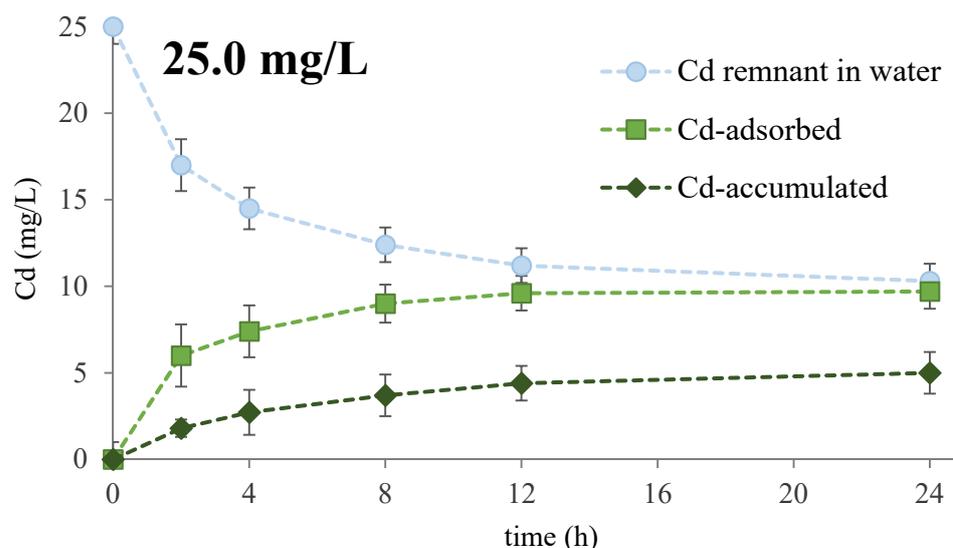

Figure 1. *Cont.*

Figure 1. Metal removal (●), adsorption (■), and intracellular accumulation (♦) kinetics in living *Oscillatoria* sp. during 24-h exposure at 5.0 or 25 mg/L of Cd. Data are mean ± SD of three independent experiments (*n* = 3).

Additionally, Cd removal from water fits a second-order reaction model (Table 1), suggesting that the entire bioprocess depends not only on the metal concentration, but also on the number/type of metal-binding sites in the microalgal biomass, among other potential metabolic features.

Table 1. First- and second-order kinetic parameters obtained for Cd removal by *Oscillatoria* sp.

Cd (mg/L)	First-Order Kinetics		Second-Order Kinetics	
	K_1 (1/h)	R^2	K_2 (L/mg h)	R^2
5.0	0.0712	0.9237	0.0242	0.9981
25.0	0.0597	0.8329	0.0038	0.9815

In fact, a biphasic nature of Cd elimination can be clearly observed in Figure 1, with an initial rapid phase that occurs in the first 4 h, and a slower one from 6 h onwards that reaches a plateau between 12–24 h of metal exposure. This non-linear Cd removal kinetics could be explained by the fact that living *Oscillatoria* sp. utilizes two principal mechanisms for metal elimination. The fastest component of metal uptake is Cd adsorption on the cyanobacterial cell outer membrane, while the second most important mechanism for Cd removal is metal accumulation within the cells (Figure 1). Moreover, the amount of accumulated Cd increased with time and was higher as the initial concentration of metal in the sample increased, suggesting the existence of some metabolic or physiological responses in *Oscillatoria* sp. that depends on the metal concentration, and to a lesser extent, on the time of exposure to the toxic metal.

In general, metal accumulation in living organisms involves a series of complex biologically-regulated processes, such as metal translocation by transmembrane proteins (e.g., porins) and ionic channels, and the posterior interaction with cysteine- and glutamic acid-rich peptides, such as glutathione, phytochelatins, and metallothioneins [19,36]. These peptides are able to form stable metal–protein complexes, thus reducing the deleterious effect of toxic metal ions on living cells [37]. The occurrence of metallothioneins and their importance for heavy metal detoxification were reported in several organisms including plants, yeast, bacteria, mollusks, cyanobacteria, and mammals [38]. In cyanobacteria, metallothioneins were first identified in cells adapted to grow in elevated levels of Cd or Zn [19]. Moreover, [39] reported the Cd-induced expression of a novel metallothionein (BmtA) in *Oscillatoria brevis* and its importance for metal detoxification. In addition, these

authors suggested that *O. brevis* utilizes metallothionein as a primary heavy metal resistance mechanism to confer Cd tolerance to cells. A similar response might be used by other *Oscillatoria* species to deal with heavy metal toxicity; however, more experimental evidence is needed to support this hypothesis.

Our results showed that an increase in Cd accumulation was detected when metal concentration increased from 5.0 to 25.0 mg/L, suggesting the induction of some response mechanisms (e.g., synthesis of metallothioneins, increased metal-storage/retention in vacuoles, etc.) involved in Cd bioaccumulation by *Oscillatoria* sp. However, the proportion of metal adsorbed was always higher than the amount of metal accumulated.

It was described that metal adsorption to cyanobacterial biomass occurs by a number of different physico-chemical interactions involving ionic bonds, cationic exchange, and chemical chelation. The presence of polysaccharides, proteins, and lipids on the surface of their cell walls containing different functional groups such as amino, hydroxyl, carboxyl, sulfate, and phosphate might act as binding sites for cationic metals [30]. In addition, the presence of negative charges (e.g., uronic acids) in cyanobacterial exopolysaccharides was proposed to play an important role in the sequestration of cations, a phenomenon of major interest for metallurgical industry wastewater treatment [19]. Therefore, a change in the functional groups of *Oscillatoria* sp. surface is a key point to understanding the metal binding mechanism in these photoautotrophic microorganisms.

In this study, functional groups on the surface of the cyanobacterial biomass responsible for Cd adsorption were determined by using FTIR-ATR spectroscopy, with and without Cd exposure. As can be appraised in Figure 2, several major bands at 3260, 2928, 1730, 1628, 1510, 1450, 1387, 1231, 1146, and 1026 cm^{-1} were observed in the spectrum of non-treated *Oscillatoria* sp. biomass.
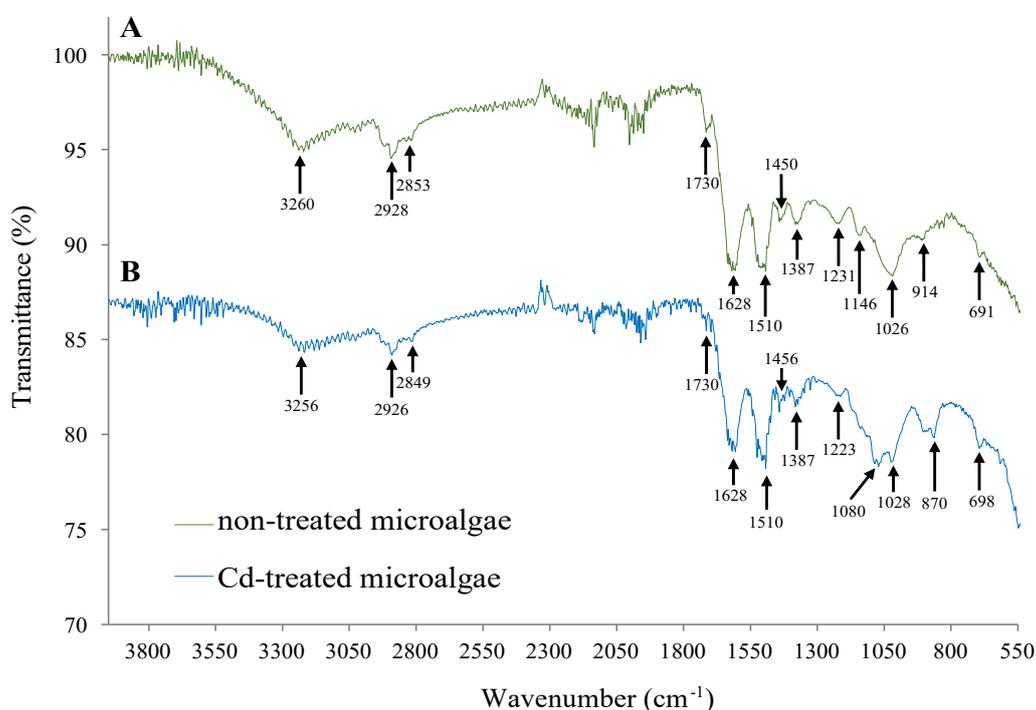
Figure 2. FTIR-ATR spectra of *Oscillatoria* sp. grown under control conditions (**A**) or after 24-h Cd exposure (**B**).

The broad band at 3500–3050 cm^{-1} was characteristic of the O–H and N–H groups [30], whereas the bands at 2928 and 2853 cm^{-1} refer to the asymmetric and symmetric vibration of methylene (CH_2), respectively [28]. The peaks at 1730 cm^{-1} correspond to the C=O bond. The bands within the region of 1500–1000 cm^{-1} were characteristic of the ester group. Several peaks were obtained in this region, indicating the stretching vibrations. Other bands of the spectrum could be attributed as follows: 1628 cm^{-1} (–NH_2 group or

–C–N of amide), 1510 cm^{-1} (–N–H bending), 1387 cm^{-1} (–CH$_3$ stretching), 1231 cm^{-1} (NO$_2$ antisymmetric stretch), 1146 cm^{-1} (–C–O stretching of carboxylic acids), and 1026 cm^{-1} (–C–N and –C–C stretching vibrations) [40]. The bands <930 cm^{-1} are the fingerprint zone and include phosphate and sulfur functional groups [28].

Remarkable differences were observed in the spectra of *Oscillatoria* biomass obtained with and without Cd treatment (Figure 2). Many peaks in the spectrum of untreated cyanobacterial biomass shifted due to the presence of Cd in the medium. The shifting of peaks indicates that *Oscillatoria* sp. possess a metal binding ability, indicating the usefulness of this microalgae for treating Cd-containing effluents. In addition, some other bands showed a lower intensity because of the presence of the metal. These changes could be due to the Cd removal by the filamentous microalgae. Therefore, our results proved that different functional groups (mainly anionic ones) present at *Oscillatoria* sp. biomass such as carboxyl, phosphate, amide, amino, hydroxyl, sulfur, and others play a significant role in Cd adsorption. These results were consistent with related studies on the exposure of different microalgae to toxic metals [30,40].

Recently, [41] characterized the functional groups potentially involved in divalent copper ions (Cu^{2+}) adsorption on dried algae biomass of *Oscillatoria splendida*, by using FTIR spectroscopy. These authors described the presence of carbonyl, hydroxyl, and aliphatic chains acting as ligands for Cu binding. In the present study, the FTIR–ATR spectra obtained for *Oscillatoria* sp. biomass (Figure 2) with and without Cd-exposure were closely related to that reported by [41], suggesting that the high content of carbohydrates and lipids with negatively charged groups (mainly carboxyl and phosphate) present in the cyanobacterial biomass was the most relevant factor in determining Cd binding.

Related to this, [15] reported that Cd biosorption was maximal at a pH near neutrality, due to an increase in the number of negatively-charged ligands, which favored the binding of cationic metals, such as carboxyl and phosphate groups of lipopolysaccharides and phospholipids that were present in the outer membrane of *Oscillatoria*. Moreover, at more alkaline pH values (e.g., pH > 8), Cd solubility could be lowered due to the formation of metal hydroxides. On the other hand, in acidic conditions (pH < 5), carboxyl and phosphate groups were closely linked to H$^+$ ions, thereby making these sites unavailable for Cd cations. Therefore, in order to ensure an active biological uptake by the free-living microalgae, media pH regulation is another important parameter to be considered since it not only affects cell viability and acid–base behavior of the metal binding sites, but also metal chemistry and bioavailability.

As mentioned before, the efficiency of Cd elimination by *Oscillatoria* sp. was negatively affected by the increase in Cd concentration from 5.0 to 25.0 mg/L. However, it is worth noting that at higher Cd levels of 50 mg/L, a greater metal removal efficiency was evidenced (>99%), probably due to an increasing cell damage (*data not shown*). This fact could be associated with the exposure of additional metal-binding sites that enhanced Cd biosorption properties of the dead biomass, since non-living cells proved to be more efficient than living ones for metal elimination [15]. However, when microalgae were used as biosorbents (i.e., dead or inactive biomass) for removing metal ions, their low growth rates and cell densities made the biomass production very costly to be further used as inactive biomass for wastewater treatment. In contrast, the removal of metal ions by live microalgae cultures could overcome this disadvantage. In addition, a bioremediation of some nutritional components that frequently contributed to the eutrophication of aquatic ecosystems always occurred simultaneously during microalgae cultures [42].

Finally, one of the major hurdles in the removal of heavy metals by microalgae cultures is the high cost associated with the harvest of tiny cells from diluted cultures. A cost-effective strategy for removing microbial cells from diluted broth is gravity sedimentation facilitated by flocculation, particularly by the self-flocculation properties of some microbial species. Thus, compared to other regular cyanobacteria species, flocculating *Oscillatoria* sp. presents as an excellent candidate to be further explored for improved heavy metal

tolerance, with the aim of applying it in metal-polluted wastewater management, due to the cost-effective sedimentation of these self-flocculating microorganisms.

3.2. Evaluation of Cd Toxicity in Oscillatoria sp.

Cyanobacteria are frequently challenged by toxic metals that have no function as nutrients. Due to their photoautotrophic lifestyle, cyanobacteria evolved to deal with toxic reactive oxygen species (ROS) produced by their metal-rich photosynthetic apparatus [19]. In the present study, *Oscillatoria* sp. showed different metabolic and physiological responses to Cd challenge. As can be seen in Table 2, Cd toxicity in *Oscillatoria* sp. live cells involves the generation of ROS, as shown by the increment in the levels of malondialdehyde (MDA). After 24-h of metal exposure, the content of MDA significantly increased by about 15% ($p < 0.05$) when the cells were treated with 5.0 mg/mL of Cd, in comparison to the control untreated group, and up to 28% ($p < 0.05$) with the highest tested metal concentration of 25.0 mg/mL. These results might be explained by the increase in Cd bioaccumulation by microalgae, showed in Figure 1.

Table 2. Dehydrogenase activity; and concentrations of malondialdehyde (MDA), total protein, photosynthetic pigments, and soluble carbohydrates in *Oscillatoria* sp. cells after 24-h Cd exposure.

Parameters	Cd (mg/L)		
	0.0 (Control)	5.0	25.0
MDA content (μmol/mg)	7.35 ± 0.31 [a]	8.42 ± 0.16 [b]	9.41 ± 0.04 [c]
DHase activity (UA/mg)	17.7 ± 0.1 [a]	14.6 ± 0.1 [b]	14.4 ± 0.4 [b]
Cell density (O.D.$_{560\ nm}$)	0.567 ± 0.023 [a]	0.558 ± 0.027 [a]	0.528 ± 0.040 [a]
Total proteins (mg/L)	118.9 ± 6.7 [a]	118.0 ± 10.0 [a]	113.8 ± 6.5 [a]
chl-a (μg/mg)	53.6 ± 3.6 [a]	53.7 ± 1.7 [a]	51.2 ± 3.4 [a]
Carotenoids (μg/mg)	139.3 ± 7.4 [a]	119.8 ± 2.1 [b]	117.6 ± 6.0 [b]
Carbohydrates (μg/mg)	710 ± 85 [a]	879 ± 36 [b]	909 ± 21 [b]

MDA, malondialdehyde; DHAse, dehydrogenases. Results are presented as mean ± S.D. ($n = 3$). Different lowercase letters in the same row represent significant statistical difference ($p < 0.05$) between metal concentration (e.g., "a" and "b" are statistically different from each other).

Metal toxicity is based on its chemical properties, and in general, promotes the production of ROS, the inactivation of enzymes, or the displacement of the normal metal cofactors in some cyanobacterial metalloproteins [19]. It was described that Cd ions are able to induce oxidative stress at similar doses to the ones used in the present study in related microorganisms, such as *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, and *Spirogyra setiformis* [40,43,44]. Our results agreed with these observations; however, to the best of our knowledge, this was the first description of the Cd-induced oxidative stress in free-living *Oscillatoria* sp. cells.

The MDA content is considered an index of lipid peroxidation and a high level of MDA accumulation from peroxidation of unsaturated fatty acids can cause serious damage on nucleic acids and proteins [32]. In addition, ROS reaction with lipids and proteins might cause membrane damage, biomolecule degradation, and enzyme inactivation [19]. In fact, a decrease (~18%, $p < 0.05$) in dehydrogenase (DHase) activity was detected in *Oscillatoria* sp. cells exposed to Cd, with respect to the untreated control cells, after a 24-h metal exposure (Table 2). The reduction of tetrazolium salts, such as TTC, to red-colored formazan through degradative dehydrogenases, is a widely accepted method for detecting the physiological state of several organisms, including microalgae cultures [33,45]. Further, as TTC is a metabolic dye, suboptimal growth or stressful conditions (e.g., nutrient limitation, toxicant presence, etc.) could lead to inefficient TTC reduction by cells [46]. Therefore, the quantity of intracellular formazan product, estimated by its absorbance at 485 nm, could be used as a surrogate marker of pollutant cytotoxicity [47]. Hence, the results shown in Table 2 suggest that *Oscillatoria* sp. is partially affected by Cd exposure and this might be related to metal-induced oxidative damage.

However, it is noteworthy that cyanobacterial DHase activity decreased to a similar extent when *Oscillatoria* sp. was treated with 5.0 mg/L of Cd or with a metal concentration 5 times higher (i.e., 25.0 mg/L). In addition, no changes in cellular density (O.D.$_{560nm}$), protein content, and chlorophyll *a* (*chl-a*) concentration were observed after 24-h metal exposure between control cells and Cd-treated cyanobacteria (Table 2). These results strongly suggest that *Oscillatoria* sp. might develop different enzymatic and non-enzymatic intracellular antioxidant responses to depress the oxidative stress induced by the metal. Additionally, it is important to mention that cellular reduction of tetrazolium compounds might proceed independent of ATP production [45], thus the observed decrease in the DHAse activity in *Oscillatoria* sp. is not necessarily related to an ATP depletion in the cells. However, as DHase activity is also related to cell viability, it should be appropriate to further evaluate cyanobacteria growth after long-term Cd exposure.

As mentioned before, *Oscillatoria* sp. seems to trigger some intracellular strategies to deal with Cd-induced oxidative damage. As can be appraised in Table 2, Cd exposure in *Oscillatoria* sp. interferes with the normal metabolism of carotenoids, since a significant reduction (~15%, $p < 0.05$) in the total carotenoid content was observed in culture cyanobacteria exposed to 5.0 mg/L or 25.0 mg/L of Cd for 24 h. Carotenoids are known to play important roles as antioxidants and accessory light-harvesting pigments. These compounds are essential to photosynthesis, acting as secondary pigments. They are also pro-vitamin factors, and are involved in free radical elimination [48]. Reactive oxygen species (ROS) can oxidize carotenoids, leading to a variety of oxidized products, including aldehydes, ketones, endoperoxides, and lactones. Some of these are reactive electrophile species that are bioactive and can induce changes in the gene expression, leading to acclimation of cells to stress conditions [49]. Furthermore, carotenoids quench excess excitation energy to protect chlorophyll molecules (the main pigments responsible for collecting solar radiation during the photosynthetic process) from oxidative damage preserving the ATP levels [50]. Therefore, our results suggest that *Oscillatoria* sp. might improve its antioxidative defense system under the metal stressful conditions through a carotenoid-mediated ROS quenching. Related to this, it is noteworthy that the *chl-a* content between the control and Cd-treated *Oscillatoria* sp. cells did not statistically change, probably due to a faster hydrolysis of carotenoids, compared to *chl-a* in the cells under metallic stress (Table 2).

Moreover, the cyanobacteria culture did not show a marked reduction of their cell density (as a surrogate marker of biomass growth) and total protein content, as compared to the control group, after a 24-h metal exposure (Table 2). In this context, [42] recently proposed that the formation of photosynthetic pigments, in particular *chl-a*, synchronized with the growth of the microalgal cells, could be used as an indicator for evaluating the removal efficiency of metal ions from polluted water.

Besides its impact on cyanobacterial oxidative stress, DHAse activity, and carotenoid content, Cd exposure also affected carbohydrate production by the filamentous microalgae. As shown in Table 2, the content of soluble carbohydrates in *Oscillatoria* sp. significantly increased ($p < 0.05$) at both tested metal concentrations (i.e., 5.0 and 25.0 mg/L). These results might be explained by a reduction in carbohydrate utilization as a consequence of a lower rate in carbon assimilation caused by the reduction in the total carotenoid content. Further, the environmental stressful conditions caused by Cd exposure could induce the mobilization of soluble sugars where required, in order to preserve the osmotic homeostasis of the cyanobacterial cells, since these compounds not only act as structural cellular constituents, but also as intracellular signaling molecules involved in the regulation of metabolic processes associated with growth, ATP production, and survival of cells. Therefore, it is possible that the stress caused in *Oscillatoria* sp. due to the intracellular accumulation of Cd prompts an increase in the amount of soluble carbohydrates as a protective mechanism, in order to counteract the oxidative damage, since these biomolecules play a very active role in cell energy management. A similar behavior was reported in plants exposed to heavy metal stress [51–53], thus, it is possible that a similar mechanism could be used by photosynthetic microalgae.

Collectively, these data suggest that Cd-challenged *Oscillatoria* sp. might trigger an integrated reprogramming of their carotenoid and carbohydrate metabolism to protect, on one hand, the photosynthesis (i.e., preserve *chl-a* content), which normally provides ATP for cell growth and viability, and on the other hand, the protein synthesis, limiting in this way the poisoning incorporation of Cd into cells. Therefore, the tolerance of a cyanobacterium against heavy metal stress seems to be controlled by a complex and highly interrelated network of molecular and physiological approaches that help to counteract metal phytotoxicity. However, more evidence in *Oscillatoria* sp. is still needed at the molecular level (e.g., up- and down-regulation of genes), in order to understand these fascinating mechanisms. Additionally, large-scale performance assays and long-term metal removal studies using free-living cells are still needed to further design microalgae-based systems for industrial-scale wastewater management.

4. Conclusions

New insights on Cd removal by free-living *Oscillatoria* sp. was presented. FTIR–ATR spectroscopy revealed that distinct functional groups present at the surface of the cyanobacterial cell walls play a significant role on metal adsorption. However, *Oscillatoria* also showed a great potential for the bioaccumulation of Cd, suggesting the induction of some intracellular strategies to deal with Cd toxicity. Additionally, Cd bioaccumulation induced cell oxidative damage and harmed cyanobacterial metabolic activities, under the metal-stressful conditions. However, *Oscillatoria* sp. proved to be able to early trigger some defense mechanisms against the Cd-induced oxidative damage, such as ROS quenching by carotenoids and the increase in carbohydrates catabolism (e.g., glycogen degradation), in order to maintain an active photosynthetic and energetic machinery. Notably, no impact of Cd toxicity on cell growth, chlorophyll-*a* content, and total protein of microalgae was observed after 24-h metal exposure, even at the highest metal concentration tested (i.e., 25.0 mg/L). Therefore, our study opens the possibility of finding an equilibrium that maximizes metal removal performance with an active cyanobacterial metabolism. Hence, additional studies are needed in order to efficiently couple Cd bioremediation with *Oscillatoria* biomass growth, so as to exploit the fully metabolic potential of these photosynthetic microorganisms, aiming to achieve a rewarding and more sustainable management of industrial metal-polluted wastewater.

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