Arsenic tolerance of *Microcystis novacekii* (Komárek-Compère, 1974) and its arsenic decontamination potential

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ABSTRACT

Cyanobacteria possess metallic ion interaction properties that should be explored with the aim of recovering arsenic (As) contaminated areas. Contamination of As is an issue of worldwide concern due to the risk of human chronic intoxication and negative environmental health effects. In this study the potential decontamination of As(III) and As(V) using cyanobacteria cultures was assessed. Microcystis novacekii (Komárek-Compere, 1974) showed normal growth in concentrations of As(V) similar to those found in natural environments contaminated with As, demonstrating its resistance to As(V). Growth rates gradually decreased upon exposure to high As(V) concentrations from 600 to 5630 mg.L^{-1} while As(III) affected growth from 14.7 - 85.7 mg.L^{-1}. The As(III) EC$_{50}$ value (41.0 mg.L^{-1}) was 140-fold lower possibly due to differences in As(III) and As(V) absorption pathways. Upon exposure to 14.7 mg.L^{-1} As(III), 21.2% of As was removed from culture medium. The absorption capacity (12000 mg.kg^{-1}) remained constant with increasing As(III) concentrations in a dose independent effect. The potential of M. novacekii for As decontamination was demonstrated in this study. This microorganism is recommended in As bioremoval studies due to its autotrophic-mixotrophic growth, low nutritional requirements and high As(III) absorption capacity.

Keywords: arsenic, cyanobacteria, bioaccumulation, toxicity, growth rates.

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INTRODUCTION

Arsenic (As) is an element that occurs in natural waters ranging from typical concentrations less than 10 µg.L^{-1} to values above 50 µg.L^{-1} in arseniferous groundwater [1]. Anthropogenic dispersal of this toxic element is mostly a result of water drainage in mining areas in arseniferous deposits [2] or when water from aquifers with high concentrations of dissolved As is used for public supply [3]. The risk of As exposure is an issue of worldwide concern [1, 4]. Chronic intoxication by this element has been reported in several countries (e. g. Bangladesh, Vietnam, China, Argentina, Chile) where As in water exceeds safety limits in some localities [5, 6, 7]. On the aquatic ecosystem As pollution can affect the equilibrium and coexistence of aquatic organisms. Furthermore, the capacity of As accumulation by aquatic primary producers is determinant for its biomagnification in the trophic chain [8].

Using microorganisms as a tool for removal of contaminants such as metals and metalloids is a low-cost process known as bioremediation [9]. Bioremediation technologies have a promising potential to contribute eco-friendly manner. These comprise high-efficiency techniques for heave metal removal in from dilute solutions, in addition, they could provide metal recovery[10]. The accumulation and concentration of pollutants from aqueous solutions by the use of biological materials [such as aquatic macrophytes biomass[11, 12, 13] or microbial biomass [14, 15, 16], facilitating the recovery and/or environmentally acceptable disposal of the pollutant is termed as "bioremoval" [17].

The possibility of using clean technologies for environmental decontamination has spurred the search for resistant organisms that are capable of biotransforming toxic elements such as As. The possibility of using clean technologies for environmental decontamination has spurred the search for resistant organisms that are capable of biotransforming toxic elements such as As [18]. Arsenic metabolism by cyanobacteria has mainly been studied in water-bloom-forming species from eutrophic lakes [19, 20, 21].

Cyanobacteria usually dominate in waters contaminated with As [21]. These mixotrophic organisms occupy a unique taxonomic position, combining autotrophic metabolism, common to eukaryotic plant cells, with a heterotrophic metabolic system proper to bacteria [22]. Their capacity to tolerate adverse conditions is due to adaptive characteristics such as nitrogen fixation, chromatic adaptation and nutrient storage in cytoplasmic inclusions [23]. All these features makes cyanobacteria an interesting group to study its potential of As immobilization and water decontamination.

The cyanobacterium *Microcystis novacekii* (Komárek - Compère, 1974) was selected for this study for its wide environmental distribution and for being able to produce a mucilaginous sheath with a relevant role on metal adsorption [24, 25]. The *Microcystis* genus is cosmopolitan and commonly found in eutrophic lakes. However, studies of *M. novacekii* on toxicity and accumulation of As are not found in the literature. Therefore, the aim of this study was to evaluate As(III) and As(V) toxicity for *M. novacekii* and its potential for As decontamination in culture medium in laboratory conditions. The decontamination of As by *M. novacekii* may indicate the relevant role of this organism in As removal from aquatic ecosystems and its potential to mitigate this major source of environmental impacts worldwide.

MATERIALS AND METHODS

Cyanobacteria culturing

M. novacekii was isolated from water samples collected at Dom Helvécio Lake (19°46'419"S; 42°35'595"W), located in the Rio Doce State Park, the largest remnant of the Atlantic Forest in the State of Minas Gerais, Brazil. The cyanobacteria strain has been maintained in the algae culture bank in the Laboratory of Limnology, Ecotoxicology and Aquatic Ecology of the Universidade Federal de Minas Gerais.

Toxicity and As bioaccumulation experiments

M. novacekii was cultivated in Acibenzolar-S-Methyl medium (ASM-1) prepared with ultrapure water (Milli-Q), modified by the addition of 3-(N-morpholino) propane sulfonic acid buffer (Sigma-Aldrich, St. Louis, MO, USA) (750mg.L^{-1}) and adjusted to pH 7.

Cultures (150 mL) were prepared in 250 mL Erlenmeyer flasks, under continuous light with a white cold fluorescent lamp (60 µmol.m^{-2}.s^{-1}) and constant agitation (70 rpm) with a rotary

agitator (Marconi 140, Piracicaba, SP, Brazil) in temperature of 20±1°C. The salts disodium hydrogen arsenate heptahydrate ($Na_2HAsO_4.7H_2O$) and sodium (meta) arsenite ($NaAsO_2$) (Sigma-Aldrich, St. Louis, MO, USA) were used to prepare stock solutions in concentration of 15000 mg.L^{-1} of As(V) and 10000 mg.L^{-1} of As(III), respectively, which were added to 150 mL of *M. novacekii* culture in log phase with a cell density of 10^6 cells.mL^{-1} to obtain two concentrations series: 0.0, 0.8, 8.0 and 80.0 mg.L^{-1} of As (V) (series A) and 0.0, 600, 1050, 1840, 3220 and 5630 mg.L^{-1} of As (V) (series B). For As(III) the series of final concentrations was 0.0, 14.7, 26.5, 47.6 and 85.7 mg.L^{-1}. All experiments were performed in triplicate. The effective concentration of As(III) and As(V) that led to 50% of growth inhibition (EC$_{50}$) for *M. novacekii* was determined according to OECD 201 protocol. The As concentrations were determined in previous tests arranged in geometric series as recommended in EC$_{50}$ tests [26].

Analytical procedures
After exposure to As for 0, 96, and 192 h, aliquots of 10 mL cultures from the experiment previously described were centrifuged (Sigma 4k15, Germany) at 3000 rpm for 15 min, at 15°C. The supernatant were transferred to new tubes, the pH was adjusted to 2.0 with 1 mol.L^{-1} HCl (37%, Merck KGaA, Darmstadt, Germany) and the aliquots were stored at 4°C until further analysis [27]. The pellets were re-suspended in culture medium and rinsed three times with deionised water, followed by mineralization in teflon vessels with 3 mL HNO_3 (65%, Sigma-Aldrich, St. Louis, MO, USA) and 1 mL hydrogen peroxide 30% (Sigma-Aldrich, Buchs, Germany) in microwave oven (ETHOS 1–Advanced microwave digestion system/Model Milstone) at 200°C and 45 bar for 30 min. After cooling to room temperature the final volume was adjusted with water to 25 mL. As concentrations were determined in the cyanobacteria biomass (pellets) and also in the supernatant to quantify the amount of As remaining in the culture medium. The analysis were performed by inductively coupled plasma optical emission spectroscopy, optima 4300 DV Perkin Elmer, Shelton, USA.

Adsorption of As(III)
To evaluate As(III) adsorption by cyanobacteria cell wall, a new experiment in shorter period of exposure (2 h) was carried out. Aliquots of 100 mL of the culture in the logarithmic growth phase (10^6 cell.mL^{-1}) were prepared in the same condition described (subsection: Toxicity and As bioaccumulation experiments) were exposed to As(III) in a shorter period of time (2 h) at concentrations of   2.5, 5.0, 7.5, 10.0, 12.5 and 15.0 mg.L^{-1}. Total As concentrations in supernatants and pellets were determined as described (Analytical procedures). Only As(III) adsorption experiments were performed due to the higher As(III) removal capacity from the culture medium compared to As(V).

Data analysis
Growth rates were calculated according to the following equation:

$$\mu_{i-j} = \ln X_j - \ln X_i/(t_j - ti)\,day^{-1}$$

Where: μ is the average specific growth rate from the time i to j in days
X_i is n° of cells mL^{-1} at time i.
X_j is n° of cells mL^{-1} at time j.

Growth inhibition was obtained from the equation:
$$\%I_r = [(\mu_c - \mu_t)/\mu_c]\,x\,100$$
Where: % I_r is the percentage of inhibition of the specific growth rate
μ_c is the average growth rate in the control group.
μ_t is the average growth rate in replicas of the tests.

The As(V) and As(III) bioaccumulation were calculated by equation:
$$\%As_b = 100 - \frac{As(Xi) - As(Xm)}{As(Xi)}\,x\,100$$
Where: %As_b is the percentage of arsenic bioaccumulated by the cyanobacteria biomass;
As(Xi) is the arithmetic mean of arsenic concentration added to the aqueous medium at time i;

As(Xm) is the arithmetic mean concentration of arsenic found in the cell biomass after a period of time.

The EC_{50} for As(V) and As(III) were calculated by linear regression equation: $y = -142.3 + 22.2x$ and $y = -145.0 + 52.5x$, respectively. The homogeneity of variance was tested using Levene's test.

Growth rates in each concentration were compared to control into the same As(III) or As(V) geometric series by a one-way analysis of co-variance (ANCOVA). Differences were considered significant when $p < 0.05$. The analyses were performed in the software Statistica 8.

RESULTS

As(III) and As(V) toxicity

At concentrations of 0.08 to 80.0 mg.L^{-1} (series A) As(V) did not affect the growth rates of *M. novacekii* ($p > 0.05$) (Figure 1a). It is important to reinforce that these concentrations are similar to those found in water contaminated with As(V). At concentrations of 600 to 5630 mg.L^{-1} (series B) As(V) growth decrease, due to the As toxicity what can be observed at concentrations higher than 1050 mg.L^{-1}($p < 0.05$) (Figure 1b). Experimental conditions were the same in both series. *M. novacekii* growth decreased significantly when exposed to As(III) (47.6 and 85.7 mg.L^{-1}). Significant growth reduction started at 47.6 mg.L^{-1} ($p < 0.05$) markedly after the first 24 h followed by a gradual recovery after 48 h (Figure 1c).
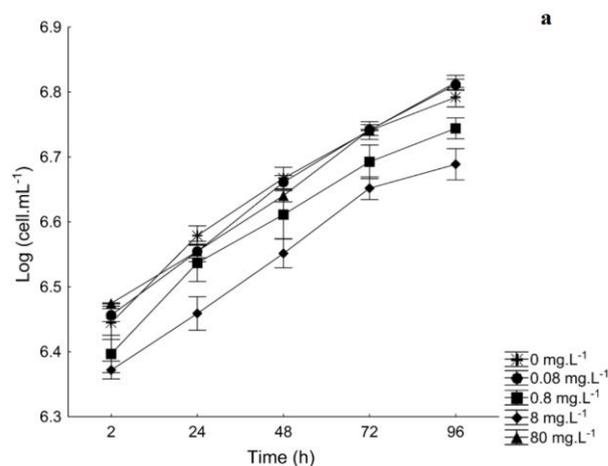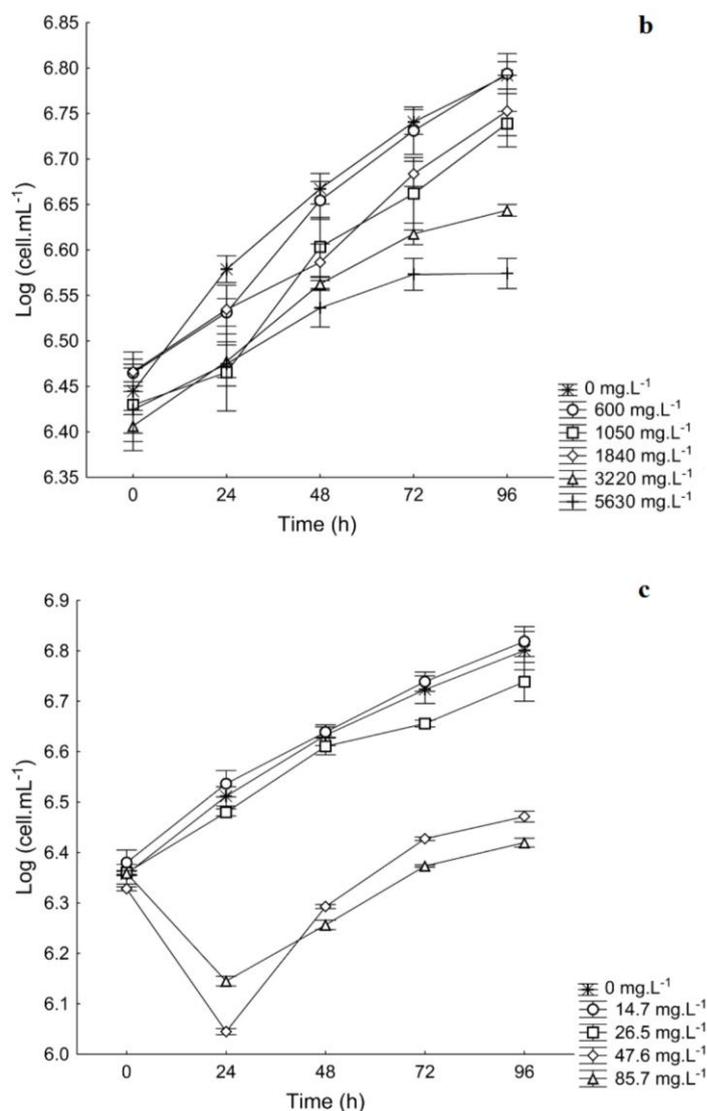
Figure 1. Growth of *M. novacekii* after 96 h of exposure to As(V); a, series A: 0 to 80.0 mg.L-1; b, series B:0 to 5630 mg.L-1; c, As(III) 0 to 85.7 mg.L-1.

The acute toxicity tests showed that the concentration of As(V) that effectively reduced the growth rate by 50% (EC_{50}) was 5810 mg.L^{-1} (Fig. 2a). While the EC_{50} for As(III) was 41.0 mg.L^{-1} (Fig. 2b). The concentrations of As(III) or As(V) in the supernatant remained constant during the toxicity test period (96 h).
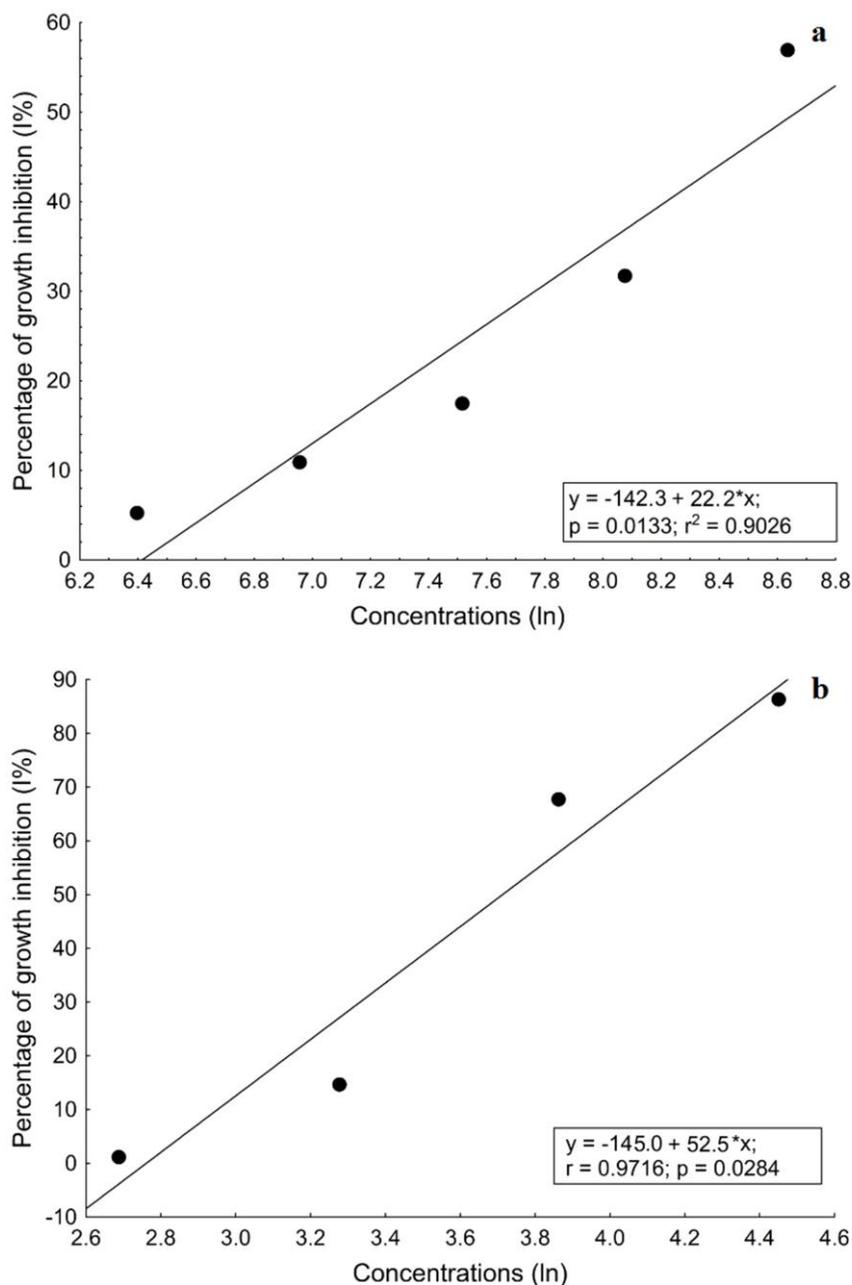
Figure 2. Percentage of growth inhibition for *M. novacekii* after 96 h of exposure to As(V) (a) or As(III) (b) at increasing concentrations.

Bioaccumulation of As by *M. novacekii*

Table 1 gives a final balance (%) after 192 h of As(V) or As(III) added to cultures. The As(V) in biomass ($mg.kg^{-1}$) increased with the concentrations of As in the culture medium reaching a maximum of 22500 $mg.kg^{-1}$. However, after 192 h, the initial As concentration in culture medium was very high compared to the absolute amount of As removed by the biomass and, therefore more than 97% of the initial As concentration remained in culture medium. As(V) in *M. novacekii* biomass ($mg.kg^{-1}$) showed different values($p<0.05$), such as: 7.7±0.5, 9.7±0.5 and 22.5±5.0 $mg.kg^{-1}$ in the three highest concentrations (1840, 3220 and 5630 $mg.L^{-1}$), respectively. Otherwise, at the lowest concentration tested (0.08 $mg.L^{-1}$), 12% of the As(V) in culture medium was removed, with bioaccumulation of 40 ± 20 $mg.kg^{-1}$ in *M. novacekii* biomass.

The As(III) maximum removal from the culture medium was 21% after exposure to 14.7 mg.L^{-1}, indicating that *M. novacekii* has a higher decontamination potential for As(III) than As(V). Increasing concentrations of As(III) from 14.7 to 85.7 mg.L^{-1} did not affect the decontamination of the metalloid by the cyanobacteria, which remained between 12600±200 and 12900±200 mg.kg^{-1} As(III) in biomass of *M. novacekii*, however those concentrations adversely affected the growth of the cyanobacteria.

Table 1. Initial As concentrations (mg.L^{-1}) and final balances (%) after 192 h of As(V) or As(III) added to cultures.

Nominal [As][a]		Actual initial [As][b]	As (%) removed by the biomass	As remaining in the culture medium (%)	[As] in biomass (mg.kg^{-1})
As(V)	0.08	0.07 ± 0.02	12.1	83.8	40 ± 20
	0.8	0.8 ± 0.01	2.0	98.0	60 ± 20
	8.0	8.6 ± 0.02	0.5	98.8	180 ± 30
	80.0	79.6 ± 7.0	0.8	97.8	2390 ± 90
	600.0	597 ± 10	0.7	97.5	17800 ± 7300
	1050.0	1038 ± 23	0.4	97.6	17900 ± 1800
	1840	1857 ± 61	0.1	97.9	7700 ± 500
	3220	3386 ± 100	0.1	97.8	9700 ± 500
	5630	5790 ± 190	0.1	97.9	22500 ± 5000
As(III)	14.7	14.8 ± 0.3	21.2	76.6	12600 ± 200
	26.5	28.4 ± 0.4	11.4	88.4	13000 ± 500
	47.6	49.9 ± 1.0	6.4	92.1	12700 ± 200
	85.7	105.6 ± 0.3	3.1	93.3	12900 ± 200

[a]Concentration expected.
[b]Concentration measured.
Values in column 1 and 2 are means ± standard errors.

Adsorption of As(III) in cell surface of *M. novacekii*

After 2 h of exposure to As(III) (Table 2) 440 mg.kg^{-1} was adsorbed at the maximum concentration of 15 mg.L^{-1}. This indicates that most of the As fraction removed from the culture medium after 192 h (showed in Table 1) was not physically attached to the cyanobacteria cell wall but has been bioaccumulated as demonstrated in Table 2.

Table 2. As(III) adsorbed by M. *novacekii* biomass after 2 h of exposure.

Tests (mg.L^{-1})	As in biomass (mg.kg^{-1})
2.5	210
5.0	260
7.5	310
10.0	330
12.5	350
15.0	440

DISCUSSION

The EC_{50} for As(V) was 140-fold higher than EC_{50} for As(III) for *M. novacekii* (Fig. 2). The high differences in the As(III) and As(V) toxicity reported in this study corroborate those found with other cyanobacteria species. *Anabaena doliolum* presented EC_{50} values of 4345 mg.L^{-1} for As(V) and 824.12 mg.L^{-1} for As(III) [28]. The growth rate of *M. aeruginosa* started to decrease at concentrations higher than 0.75 mg.L^{-1} of As(III) and 75 mg.L^{-1} of As(V) [29]. The sensitivity to As varies greatly with As oxidation state and also with the cyanobacteria species, suggesting the potential effects of this element on the selection of species resistant to As in contaminated environments.

A decrease in growth rates and subsequent recovery was observed during the first 24 h markedly at As(III) concentrations above 40 mg.L^{-1} (Fig. 1c). This is very likely an adaptive behavior which allows acclimation in the presence of the toxic agent. This behavior was also observed in experiments using *Anabaena* sp. PCC7120 under As stress together with reduction in carbon fixation, nitrogenase activity and chlorophyll content [30]. It is known that intracellular As(III) increases the concentration of reactive oxygen species without stimulating the antioxidant system in cyanobacteria, resulting in toxic effects [28]. The effects of extracellular As(III) on cell membrane integrity are so far poorly investigated. *M. novacekii* displayed capacity for adaptation to As(III) and As(V) and therefore, this organism has the potential to dominate environments contaminated with this metalloid.

Various As chemical species can be found in the aquatic environment as a result of biotransformation by microorganisms [15]. As(V) generally predominates in water under aerobic conditions [1]. Therefore, the effects of a wide range of As(V) concentrations near environmental levels on cyanobacteria growth were evaluated. In series A, growth inhibition was not observed (Fig. 1a) possibly because As was not absorbed at levels sufficient to disrupt cell metabolism and most of the As remained in culture medium (Table 1). This indicates a low probability of As(V) bioaccumulation in the food chain compared to other toxic elements. Rzymski et al., (2014)[31] observed in *M. aeruginosa* a bioaccumulation of 87.3% and 90.1% when 20 mg.L^{-1} of Cd and Pb where add to culture medium.

The present study verifies the theory that the removal of As from culture medium is dependent on its chemical species as shown in Table 1. As(III) and As(V) are taken up via different transmembrane transporters. As(III) may enter the cell via aquaglyceroporins a family of proteins also present in cyanobacteria responsible for the uptake of glycerol [32, 33, 34]. As(V) competes for the phosphate transporter [20, 35] when in the culture medium PO_4 is present in the initial concentration of 16.6 mg L^{-1}. *M. novacekii* displayed potential to uptake 12000 mg.kg^{-1} of As(III) after exposure to a concentration range from 14.7 to 85.7 mg.L^{-1}. Whereas in the As(V) concentration range 0.08 to 80.0 mg.L^{-1}, the maximum As absorption was 2390 mg.kg^{-1}. Interestingly, the intracellular As level remained constant with increasing As(III) concentration in culture medium revealing a saturation of absorption capacity. A similar saturation process was also observed in mosses with increasing supply of cationic metals in the medium [36]. To our knowledge, the saturation of As(III) uptake is demonstrated for the first time using a cyanobacteria (*M. novacekii*) as a test organism.

Biosorption is a physical process that in general reaches equilibrium time in less than 2 hours as demonstrated using cyanobacteria biomass to remove Cr [37] and Sb [38]. After 2 hours of exposure to As(III), the amount of As in *M. novacekii* biomass was much lower compared to the amount accumulated after 192 h (Table 1) indicating that the process of As bioaccumulation is not physical but may involve changes in gene expression, as demonstrated in *Anabaena* sp. PCC7120 after As exposure [30]. Ion exchange is the principal process of As(III) adsorption which is influenced by the As concentration, contact time, pH of aqueous medium [39] and by other elements previously adsorbed to the biomass such as iron [40]. Intracellular As can be bio-transformed in different chemical species. As(V) can be reduced to As(III) and then immobilized by interaction with sulfhydryl groups, methylated or excreted [17, 30, 33, 41]. Membrane transport and intracellular immobilization are likely to be the mechanisms primarily involved in As absorption by *M. novacekii*.

The development of technological strategies for monitoring and restoration of ecosystems contaminated by As has become a challenge for environmental scientists. In the present study, the adaptation of the cyanobacterium *M. novacekii* to high As concentration and its capacity to accumulate As was demonstrated. These findings encourage using this species in processes of

As immobilization in waste water, especially As(III), due to its higher absorption capacity compared to As(V). As water decontamination using *M. novacekii* is worth to be studied in conditions that favour its application in processes of bio-removal of metals from contaminated waters including wastewaters.

CONCLUSION

The present study verifies the theory that the removal of As from culture medium is dependent on its chemical species. *M. novacekii* presented higher efficiency of As(III) removal (21.2%) from culture medium upon exposure to 14.7 mg.L^{-1}. The bioaccumulation capacity (12000 mg.kg^{-1}) remained constant with increasing As(III) concentrations in a dose independent effect. The process of As(III) bioaccumulation is not physical but may involve changes in gene expression. As(V) toxicity was verified in concentrations 140-fold higher compared to As(III), possibly due to the lower As(V) uptake in *M. novacekii* cells what indicates lower probability of As(V) bioaccumulation in the food chain. This microorganism is recommended in As decontamination studies due to its autotrophic/mixotrophic growth, low nutritional requirements in addition to its high As(III) decontamination capacity.

ACKNOWLEDGEMENTS

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