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Arsenic tolerance of cyanobacterial strains with potential use in biotechnology

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Abstract
The arsenic content of various water bodies in Argentina is higher than the acceptable levels for human and animal uses. Cyanobacteria are widely distributed in aquatic environments and can bioaccumulate arsenic (As). This study presents the response of indigenous cyanobacteria to As(III) and As(V), including the species *Tolypothrix tenuis*, *Nostoc muscorum* and *Nostoc minutum*, previously used with biotechnological purposes. As(III) resulted more toxic than As(V) in all cases, causing cell death in the range of 5-20 mg/l. *T. tenuis* growth was sensitive to As(V) with lethal inhibition at 625 mg/l, whereas the *Noctoc* species were stimulated. EC50 values found were 73.34 mg/l for *N. muscorum* and 989.3 mg/l for *N. minutum*. Batch cultures of *N. minutum* showed improvements in both growth parameters and photosynthetic pigment content in the presence of 1,000 mg/l As(V). Increases of 66.7%, 75.5%, 40% and 20.7% in cell productivity, chlorophyll *a*, total carotenoids and C-phycocyanin respectively were observed, reaching a bioaccumulated arsenic value of 37.4 μg/g at the stationary growth phase.

PALABRAS CLAVE
Arsénico;
Toxicidad;
Cianobacterias;
Estímulo del crecimiento;
Curvas dosis-respuesta

Tolerancia a arsénico de cianobacterias con usos biotecnológicos

Resumen
El contenido de arsénico de diversos cuerpos de agua de Argentina es superior a los niveles aceptados para consumo humano y animal. Las cianobacterias están ampliamente distribuidas en los ambientes acuáticos y pueden bioacumular As. Este estudio presenta la respuesta de cianobacterias autóctonas a As(III) y As(V), incluyendo las especies *Tolypothrix tenuis*, *Nostoc muscorum* y *Nostoc minutum* utilizadas previamente con fines

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biotecnológicos. As(III) resultó más tóxico que As(V) en todos los casos, causando muerte celular en el rango de 5-20 mg/l. El crecimiento de *T. tenuis* fue sensible a As(V) con inhibición letal a 625 mg/l. Sin embargo, las especies de *Noctoc* resultaron estimuladas. Los valores de EC50 encontrados fueron de 73,34 mg/l para *N. muscorum* y 989,3 mg/l para *N. minutum*. Los cultivos batch de *N. minutum* mostraron mejoras en los parámetros de crecimiento y en el contenido de pigmentos fotosintéticos en presencia de 1000 mg/l As(V). Los incrementos observados en productividad celular, contenido de clorofila *a*, carotenoides totales y C-ficocianina fueron de 66,7 %; 75,5 %; 40 % y 20,7 % respectivamente, alcanzando un valor de arsénico bioacumulado de 37,4 μg/g en la fase estacionaria.

Introduction

Arsenic is a well known toxic element naturally present in the Earth crust, coming from volcanic rocks or from anthropogenic sources such as industries, agriculture, acid mine drainage and domestic wastes[16,25]. Its toxicity depends on the total concentration of the chemical species, with inorganic compounds being more toxic[5].

Over the last few decades, numerous studies have described different responses in microorganisms exposed to this metalloid, including transport system induction[27], expression levels of resistance genes[8,11], phytochelatin production[18] and variation of several biochemical parameters[2,15].

The arsenic function as a constituent element of living organisms has recently been discussed by Wolfe-Simon *et al.*[28], who described that arsenic can substitute phosphorus in macromolecules of eubacteria of the family *Halomonadaceae*.

Cyanobacteria can uptake and concentrate arsenic from the surrounding environment and arsenic-resistant genera such as *Synechocystis, Oscillatoria, Anabaena* and *Phormidium* have been reported as dominant taxa in contaminated areas of different world regions[1,2,14,21,31].

Groundwater in many regions of Argentina contains As concentrations higher than the accepted levels for human and animal uses[7]. Regional strains of cyanobacteria of the genera *Nostoc* and *Tolypothrix* have been used mainly for biotechnological purposes as a source of phycobiliproteins, Cd biosorbents and rice-biofertilizers[6,22,23]. A non-viable biomass of *Nostoc minutum* was able to eliminate Cd by biosorption in alkali-pretreated cells and the immobilized biomass in alginate beds exhibited a higher level of Cd removal in comparison with that of free cells[6].

The mass cultivation of *T. tenuis*, *N. muscorum* and *N. minutum* associated with their positive biosorption of As may contribute to increase the control and management of arsenic-contaminated waters. The aim of this work was to study the arsenic sensitivity of these strains under laboratory conditions, including tolerance screening, dose-response curves, growth measurement, variations in biochemical parameters and prospective As accumulation.

Materials and methods

Microorganisms and culture conditions

The cyanobacterial strains used were *T. tenuis* and *N. muscorum*, kindly provided by Rizobacter Argentina S.A. and *N. minutum*, a local strain isolated from brackish waters of the Province of San Luis, Argentina, identified by morphological and cultural characteristics[6].

Cyanobacterial stock cultures were maintained in Watanabe medium[23] at 30 °C in a temperature-controlled room. Continuous illumination was provided by a set of nine fluorescent lamps with a light intensity of 26 W/m^2.

Toxicity test

Toxicity tests were conducted in cyanobacterial cultures exposed to different arsenic concentrations, using sodium arsenite ($NaAsO_2$) and sodium arsenate ($Na_2HAsO_4.7H_2O$) as As(III) and As(V) solutions. Two-fold dilutions from an initial concentration of 40 mg/l of As(III) and 20,000 mg/l of As(V) contained in one ml were added to an equal volume of Watanabe medium (2X) followed by inoculation with 50 μl of cyanobacterial suspensions. The cultures were incubated during 7 days under the above mentioned conditions and growth estimation was performed by visual comparison of the blue-green apparent color (AC) of culture suspensions with and without added As. Four different apparent color standards were prepared, culture suspension with no As (1), centrifuged standard 1 resuspended in three-quarter volume (2), in half volume (3), and three-quarter dilution of standard 1 (4). The growth assessment score was: +: AC similar to standard 1, ++: AC similar to standard 2, +++: AC similar to standard 3 ±: AC similar to standard 4 and −: yellowish apparent color (cellular death). Visual comparison methods describing the apparent color are used for

analyzing the presence of algae and other suspended matter in water[24]. Results are representative of three independent experiments performed in duplicate.

Dose-response assays

Samples of wet biomass (62±6 mg) obtained from an active culture of two weeks and harvested by filtration with a sterile Buchner filter were placed into Petri dishes containing 7.5 ml of Watanabe medium (2X) plus 7.5 ml of different As(V) concentrations. The range of arsenic concentration used was related to the sensitivity of each cyanobacterium: *T. tenuis* (0.01-1,000 mg/l), *N. muscorum* (0.10-5,000 mg/l) and *N. minutum* (5-10,000 mg/l). The Petri dishes were incubated for 14 days at 30 ºC under constant illumination of 26 W/m^2. Cell growth was estimated by dry weight determinations using the whole content of each dish, centrifuged at 3,180 g for 20 min, washed twice with distilled water and dried at 96 ºC until constant weight.

The biological response relative to the control (growth with no As) was calculated with the following equations[17]:

$$\% I = (C - GR_i). 100/C$$
$$\% S = (GR_i - C). 100/C$$

Where,
% I: Percentage growth inhibition relative to control.
% S: Percentage growth stimulation relative to control.
C: Control
GR_i: Growth response [i = 0.01, 0.05,….n mg/l As(V)]

The dose-response data included: EC50 [metal concentration at which cell growth was modified by 50% with 95% confidence interval (CI) and correlation coefficient (R^2)], NOEC (metal concentration at which no effect is observed) and Emax (the maximal obtainable effect). The dose-response data were analyzed using the model of sigmoidal dose-response relationship of GraphPad Prism for Windows (version 5.01, GraphPad Software, San Diego, California, USA).

Kinetic studies

Based on the quantitative dose-response assays and the growth stimulation by As(V) observed for *N. minutum*, further kinetic studies were performed only with this strain.

N. minutum cultures were obtained in 300 ml glass columns, 37 mm i.d., containing 200 ml of Watanabe medium with or without the addition of 1,000 mg/l As(V) and air bubbled through a diffuser to improve gas mass transfer rates. Glass columns were inoculated with 175 mg of wet biomass (equivalent to 5.25 mg dry weight) coming from 2 week-old cultures and incubated at 30 ºC with continuous illumination of 26 W/m^2. Cyanobacterial growth (x) was estimated by optical density at 580 nm using the following correlation with dry weight determinations: $x_{control}$ (g/l)= 0.351 $OD_{control}$ + 0.0724 (R^2=0.987) and $x_{+As(V)}$ (g/l)= 0.410 $OD_{+As(V)}$ + 0.0604 (R^2=0.998). Morphological changes in trichomes, hormogonia, and heterocysts were evaluated by light microscopy counting 1000 cells at the end of each experiment. Cell productivity, chlorophyll *a*, total carotenoids[12], C-phycocyanin and allophycocyanin[3] were determinated after cultures reached the stationary phase. Residual and bioaccumulated arsenic in cyanobacterial biomass were detected by inductively coupled plasma atomic emission spectroscopy (ICP-AES). Prior to ICP-AES analysis, the biomass was harvested and rinsed twice with deionized water. Results are representative of at least two separate experiments in duplicate.

Data analysis

The statistical analysis of *N. minutum* growth stimulation by As(V) was based on variations in kinetic parameters and pigment levels. All the data are presented as mean ± standard deviation. Statistical analysis was carried out by the Student´s t-test using GraphPad Prism for Windows to compare the differences among the treatment and control batch cultures. Significance was indicated at 0.05 levels.

Results and discussion

The screening sensitivity of the cyanobacterial strains assayed to As showed that As(III) was more toxic than As(V) (Table 1). Although As(III) exerted the strongest toxic effect causing cell death in the range of 5-20 mg/l, differences between the strains were found, the *Nostoc* species were markedly sensitive, whereas *T. tenuis* was able to withstand a concentration of 10 mg/l before total inhibition. Conversely, the response to As(V) showed that *T. tenuis* was the most sensitive microorganism with total growth inhibition at 625 mg/l, while a notorious growth stimulation was observed with the *Nostoc* strains. *N. minutum* grew in the whole range of As concentration and *N. muscorum* showed a similar behavior up to 5,000 mg/l, with partial inhibition, cell aggregation and changes in pigmentation at 10,000 mg/l. The arsenic toxicity to freshwater microalgae is dependent on the chemical species of As. As(V) was more toxic than As(III) to freshwater green algae[4,10]; however, the opposite was found for *Chlorella vulgaris*[13]. On the other hand, a comprehensive study made with cyanobacteria present in a soil of India showed different tolerance limits to As(V); from six genera of cyanobacterial species, only *Phormidium* and *Nostoc* were able to survive at 10,000 mg/l, a concentration at which the other genera were dead[21].

The different responses to As(V) displayed by the strains in the initial screening were further analyzed by quantitative dose-response assays in terms of EC50, NOEC and Emax. Although studies on uptake, transformation and volatilization of arsenic by cyanobacteria have been performed[9,19,30], scarce reports describe the arsenic toxicity on *Nostoc* species on quantitative grounds[14,21], and to the authors' knowledge no reports exist for *T. tenuis*. *T. tenuis* was the only sensitive species to As(V) that caused growth inhibition [NOEC: 0.19 mg/l, EC50: 51.31 mg/l (95% CI: 35.86 to 73.43 mg/l , R^2: 0.96)] with cellular death at 500 mg/l (Fig. 1A).

N. muscorum and *N. minutum* showed a sigmoidal relationship of growth stimulation by As(V) with the following EC50 values: 73.07 mg/l (95% CI: 40.39-132.20 mg/l, R^2: 0.91) and 989.30 mg/l (95% CI: 739.80-1323 mg/l, R^2:

Table 1 Cyanobacterial growth tolerance to arsenic solutions using $NaAsO_2$ and $Na_2HAsO_4.7H_2O$ as As(III) and As(V) and incubated at 30 °C with constant illumination of 26 W/m² during 7 days

Cyanobacterial growth*	As(III) (mg/l)							As(V) (mg/l)						
	0	0.62	1.25	2.50	5	10	20	0	312.50	625	1,250	2,500	5,000	10,000
T. tenuis	+	+	+	+	±	±	-	+	±	-	-	-	-	-
N. muscorum	+	+	+	+	-	-	-	+	++	++	++	++	++	±
N. minutum	+	+	+	±	-	-	-	+	+	++	++	+++	+++	+++

* Growth was estimated by visual comparison of the blue-green apparent color (AC) with the following standards: 1. culture suspension with no As; 2. centrifuged standard 1 resuspended in three-quarter volume; 3. in half volumen and 4. three-quarter dilution of standard 1. The assessment score was: +: AC similar to standard 1, ++: AC similar to standard 2, +++: AC similar to standard 3, ±: AC similar to standard 4 and - : yellowish apparent color (cellular death).

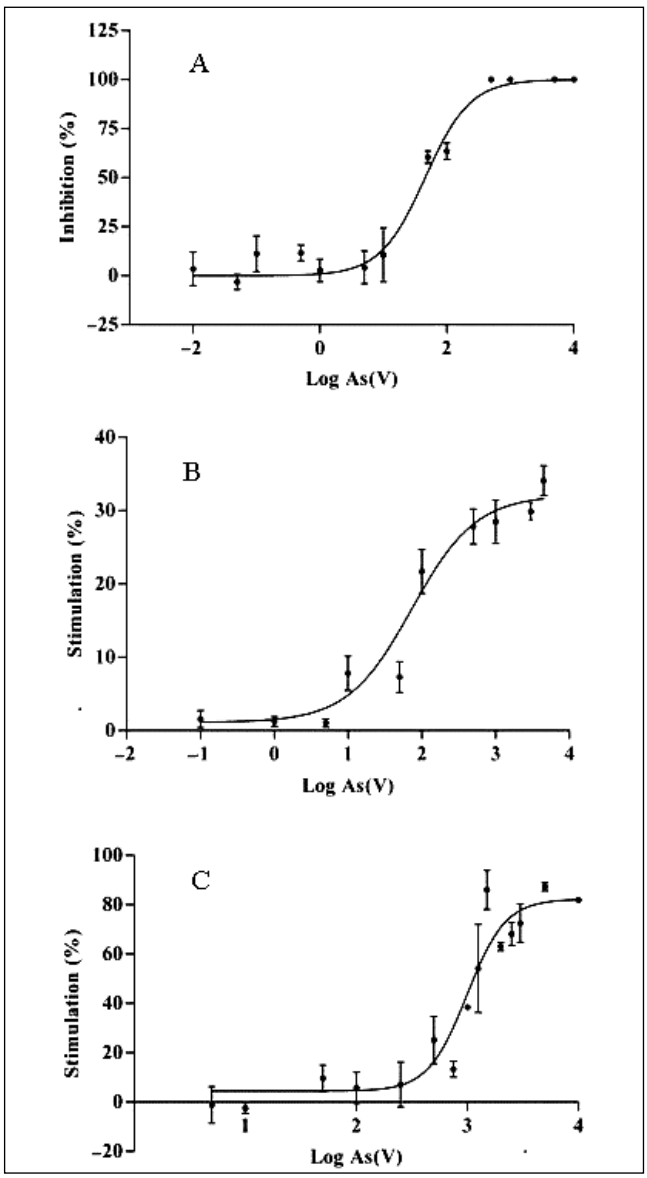

Figure 1. Dose-response curves of cyanobacterial strains to arsenic: (A) *T. tenuis*, (B) *N. muscorum* and (C) *N. minutum*. Error bars represent one standard deviation of two independent experiments in duplicate.

0.85), respectively. The growth of *N. minutum* stimulated in the presence of As(V) presented a higher EC50 value than that reported for tolerant microorganisms[28]. The *N. minutum* NOEC (70.59 mg/l) was an order of magnitude greater than that of *N. muscorum* (3.13 mg/l). The stimulatory Emax was 31.95% at 1,600 mg/l for *N. muscorum* and 82.28% at 3,100 mg/l for *N. minutum*, (Figs. 1B and 1C).

A more comprehensive analysis of the stimulatory effect of As(V) on *N. minutum* was based on the evaluation of growth kinetic and photosynthetic pigment content of batch cultures supplemented with 1,000 mg/l of As(V) (Table 2). The growth under nitrogen and carbon fixation conditions in the presence of As(V) showed improvements in kinetic parameters and cell productivity (Table 2). The lag period was shortened, with higher specific growth rate and linear growth with respect to cultures developed with no As(V). The increase in cell productivity was 1.7 times higher with no significant variations in the morphology of trichomes, hormogonia, and heterocysts ($p > 0.05$), changes that have been reported for *Anabaena azollae* cultures exposed to arsenate[20]. With the exception of C-phycocyanin, the content of photosynthetic pigments, including chlorophyll *a*, total carotenoids and allophycocyanin increased in the presence of As(V) with values that were 75.5%, 40% and 20.7% higher, respectively. Additionally the culture of *N. minutum* added with As(V) produced a more compact pellet with easy separation after centrifugation. The As(V) concentration in the supernatant decreased by 17% at the end of the culture, retaining 0.62% of As in the biomass, with a bioaccumulation of 37.4 μg/g. The overall improvement in growth kinetic parameters and photosynthetic pigments of As-supplemented cultures, at concentrations similar to the EC50, denote a possible unknown physiological role of As(V) on the nutritional state of the cultures.

The normal growth rate of cyanobacteria in the presence of arsenate 1M has been reported, which was attributed to a probable failure in the incorporation of arsenate in phosphate sufficient cells[29]. That was not the case with *Nostoc minutum* as it bioaccumulated a slight amount of As(V) in the biomass. Higher levels of As bioaccumulation were reported for species of the genera *Nostoc* and *Synechocystis*[30,31]; however, these higher values can be the

Table 2 Growth kinetic and photosynthetic pigments of *N. minutum* in the presence of arsenic. *N. minutum* cultures were obtained in Watanabe medium with or without the addition of 1,000 mg/l As(V) using glass columns and incubated at 30 °C with continuous illumination of 26 W/m^2

	Arsenic concentration (mg/l)	
	0	1,000
Kinetic parameters		
lag period (d)	2.03 ± 0.10	1.42 ± 0.05
Specific velocity (1/d)	0.21 ± 0.04	0.31 ± 0.04
Linear growth (g/l.d)	0.09 ± 0.02	0.15 ± 0.02
Final cell productivity (g/l.d)	0.06 ± 0.02	0.10 ± 0.01
Bioaccumulated arsenic (µg/g)	-	37.4 ± 2.98
Photosynthetic pigment content (mg/g)		
Chlorophyll *a*	13.36 ± 2.48	23.44 ± 3.71
Total carotenoids	0.60 ± 0.04	0.84 ± 0.05
C-phycocyanin	**105.26 ± 6.21**	**105.96 ± 8.31**
Allophycocyanin	48.1 ± 3.79	60.0 ± 4.35

All data were significantly different from respective controls with the Student's t-test ($p < 0.05$), except for numbers in bold ($p > 0.05$).

result of As bioconcentration in long-term naturally exposed cyanobacterial biomass[2].

The cyanobacterial biomass utilization with agronomical or phycoremediation purposes can be considered a green and sustainable technology. The capacity of certain strains of thriving in waters with high As concentrations widens its possible application in areas with chronic contamination to increase the control of polluted waters. However, the bioaccumulation of As in the biomass would impair its use in waste water treatment, with the risk of As entering the food chain.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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