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Kinetics and microbial structure of nitrogen cycle bacteria contained in the rhizosphere of natural wetland polluted with chromium

Cinética y estructura microbiana de las bacterias del ciclo del nitrógeno de la rizosfera de un humedal natural contaminado con cromo

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Abstract

Wetlands have been considered a feasible technology for wastewater treatment in the last decades; however, information on the kinetics and microbial structure of nitrogen cycle bacteria involved on the rhizosphere activity of natural wetlands polluted with chromium is still scarce. The goal was to evaluate the kinetic behavior of nitrification, denitrification, and ANAMMOX on rhizosphere sludge, with and without chromium, through batch cultures, as well as the microbial structure using Next Generation Sequencing (NGS). The microbial sludge was able to nitrify (3.8 ± 0.2 mg NO_3^--N/gVSS-h) and carry out the ANAMMOX (0.67 ± 0.05 mg NH_4^+-N/gVSS-h), however, denitrifying activity was not observed. Chromium inhibited the nitrifying process, and the IC_{50} obtained for the nitrifying activity was of 7.9 mg Cr^{VI}/L. ANAMMOX activity was stopped in the presence of chromium, even to the lowest chromium concentration tested. Recovery cultures showed that ANAMMOX bacteria suffered some damage by chromium presence since they required more than 5 days to recover the activity. Microbial results indicated that *Xanthomonadaceae* (17.17%), *Ignavibacteriaceae* (16.52%), *Trueperaceae* (10.66%) and *Chitinophagaceae* (10.06%) dominated in the microbial sludge, whereas Nitrosomonas and Planctomycetaceae were in lesser proportion. This research improves the understanding of bacteria behavior on natural wetlands polluted with metals.

Keywords: Chromium, ANAMMOX, nitrification, Next-Generation-Sequencing, wetland.

Resumen

Los humedales han sido considerados como una tecnología factible para el tratamiento de aguas residuales. Sin embargo, la información relacionada con el aspecto cinético y la estructura microbiana de las bacterias del ciclo del nitrógeno presentes en la actividad de la rizosfera de un humedal natural contaminado con cromo es aún escasa en la literatura. El objetivo fue evaluar la conducta cinética de la nitrificación, desnitrificación y ANAMMOX del lodo de la rizosfera, en presencia y ausencia de cromo, así como también conocer la estructura y composición microbiana a través de la técnica de Secuenciación de Nueva Generación. El lodo mostró la capacidad para nitrificar (3.8 ± 0.2 mg NO_3^--N producido/gVSS-h) y llevar a cabo el proceso ANAMMOX (0.67 ± 0.05 mg NH_4^+-N consumido/gVSS-h). Sin embargo, la actividad desnitrificante no se observó. El cromo inhibió la nitrificación, obteniendo una IC_{50} de 7.9 mg Cr^{VI}/L. El cromo inhibió completamente la actividad ANAMMOX. Las pruebas de recuperabilidad mostraron que las bacterias ANAMMOX sufrieron algún tipo de daño, ya que requirieron más de 5 días para recuperar la actividad. Los resultados microbiológicos indicaron que *Xanthomonadaceae* (17.17%), *Ignavibacteriaceae* (16.52%), *Trueperaceae* (10.66%) y *Chitinophagaceae* (10.06%) predominaron, mientras que Nitrosomonas y Planctomycetaceaese estuvieron en menor proporción.

Palabras clave: Cromo, ANAMMOX, nitrificación, secuenciación de nueva generación, humedal.

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1 Introduction

Water receiving bodies polluted with nitrogen compounds combined with metals indicate an ecological risk (Barceló-Quintal *et al*., 2012). Nitrification, denitrification, and ANAMMOX are the three biological processes involved on nitrogen cycle for the biotransformation of ammonium into N_2. In the nitrogen cycle, largest fraction of reactive nitrogen is presented as ammonium, although the presence of nitrates is also reported. Nitrification/denitrification mediated by bacteria is considered as the major route for nitrogen removal in wetlands (Li *et al*., 2014). Nonetheless, Anaerobic Ammonium Oxidation (ANAMMOX) is other route for ammonium removal, where ammonium is biotransformed to N_2, using nitrite as an oxidizing source (Saeed and Sun 2012). Nitrification is inhibited by the presence of high content of organic matter due to heterotrophic bacteria consume dissolved oxygen. Fu *et al*. (2016) in a vertical-flow constructed wetland detected genes of ammonia-oxidizing bacteria, denitrifying bacteria, and nitrite-oxidizing bacteria; suggesting that nitrification/denitrification predominated in the nitrogen removal system. Wu *et al*. (2016) identified the genera of *Zoogloea, Comamonas, Thiobacillus, Nitrosospira, Denitratisoma, Azonexus*, and *Azospira*; microorganisms that might be involved on carbon and nitrogen removal in a constructed wetland. Denitrification is restricted without the presence of organic matter, for instance, C/N ratio below 2.5 limits the denitrifying specific rates (Saeed and Sun, 2012). However, ANAMMOX activity is privileged, since the presence of organic matter inhibits the biological process (Wang and Li, 2011). Wang and Li (2011) in a constructed wetland, at low C/N ratios, *Candidatus* "Brocadia anammoxidans" with high similarity was detected, and this bacterium belongs to the order of *Planctomycetes*. Coban *et al*. (2015) observed *Brocadia* and *Kuenenia* ANAMMOX species in constructed wetlands, however, ANAMMOX activity was difficult to be measured.

Hexavalent chromium is a common pollutant introduced to water receiving bodies due to the discharge of industrial wastewaters. Chromium usually exists as trivalent (Cr^{III}) or hexavalent (Cr^{VI}) chromium. For example, Cr^{VI} has higher toxicity and it enters to the cell quickly; and once inside the cell, it is reduced to Cr^{III} that reacts with proteins and DNA (Owlad *et al*., 2009; Zarazúa-Aguilar *et al*., 2018). In constructed wetlands, the fate of chromium is by plant uptake, sorption onto porous medium and sediments (Mant *et al*., 2006; Zahoor and Rehman, 2009). It should be mentioned that there is scarce information about the effect of chromium on the kinetics and metabolic behavior of nitrogen cycle bacteria involved in wetlands. The role of bacteria in the pollutants removal in wetlands is a very significant aspect of the depuration process. The contrast between microbial activity and microbial diversity inside the wetlands is also still scarce in the literature. In this study, microbial sludge was collected from the rhizosphere of natural wetland polluted with chromium. Kinetic and metabolic behavior of nitrification, denitrification, and ANAMMOX were evaluated in batch cultures. In addition, sequencing of 16S rRNA gene (V4-V5) amplicons with Illumina Miseq was performed in order to evaluate the composition and microbial structure of sludge.

2 Materials and methods

2.1 *Biomass collection*

Ten samples of plants (*Typha* sp.) were collected of a natural wetland located in Mexico. The plants were collected with a portion of soil at the roots. The sediments were removed from the roots and washed three times with physiological solution (9 g NaCl/L). The final microbial sludge concentration quantified was 4.7 ± 0.2 g VSS/L.

2.2 *Control and recovery cultures*

Abiotic and biotic controls were undertaken in order to show evidence of chemical reactions or sorption phenomena. The sorption studies for nitrifying cultures were carried out using Cr^{VI} concentrations from 2 to 32 mg/L, whereas for anammox and denitrifying cultures were carried out using concentrations from 0.1 to 2 mg Cr^{VI}/L. Biotic controls lacking nitrite or ammonium were also included to correct for compound losses not associated with the ANAMMOX and nitrification processes. Biotic and abiotic assays were carried out by duplicate in an orbital shaker of 150 rpm at a temperature of 30 ± 0.2 °C. The Cr^{VI} concentrations were prepared by adding an aliquot of potassium dichromate stock solution ($K_2Cr_2O_7$) into synthetic water. The microbial sludge exposed to chromium

under ANAMMOX conditions was washed five times with physiological solution to remove residual chromium in order to evidence toxic or inhibitory effects in the recovery cultures.

2.3 *Nitrifying batch cultures*

Nitrifying batch cultures were undertaken in 250 mL Erlenmeyer flasks with mineral medium (60 mL) as follows (mg/L): NH_4Cl (288), KH_2PO_4 (2370), $NaHCO_3$ (9300), $MgSO_4 \cdot 7H_2O$ (600), $FeSO_4 \cdot 7H_2O$ (0.5) and $CaCl_2 \cdot 2H_2O$ (40). The experimental pH during the entire incubation period was of 7.5 ± 0.2. Batch cultures were spiked with an initial volatile suspended solids concentration of 2 ± 0.01 g VSS/L. In order to ensure aerobic conditions, the air was supplied by air pump maintaining dissolved oxygen concentration at 4 ± 0.3 mg/L. The batch cultures were evaluated with five initial concentrations of Cr^{VI} (2, 4, 8, 16 and 32 mg/L).

2.4 *ANAMMOX batch cultures*

Batch cultures were undertaken in 125 mL serological bottles containing 60 mL of basal medium. The mineral medium was prepared as follows (mg/L): NH_4Cl (288), $NaNO_2$ (246.4), $NaHCO_3$ (2500), $NaH_2PO_4 \cdot H_2O$ (57.5), $CaCl_2 \cdot 2H_2O$ (100), $MgSO_4 \cdot 7H_2O$ (200) and 1.5 mL/L_of trace elements solution. Trace elements solution contained (in mg/L): FeSO4 (5000), EDTA (5000), $ZnSO_4 \cdot 7H_2O$ (430), $CoCl_2 \cdot 6H_2O$ (240), $MnCl_2$ (629), $CuSO_4 \cdot 5H_2O$ (250), $Na_2MoO_4 \cdot 2H_2O$ (220), $NiCl_2 \cdot 6H_20$ (190) and $MgCl_2$ (500). The experimental pH was of 7.5 ± 0.3. Batch cultures were spiked with 2 ± 0.02 g VSS/L. The liquid phase and headspace were bubbled for 6 minutes with He/CO_2 (80/20, v/v) in order to eliminate the oxygen from the experimental unit. All serological bottles were sealed with butyl rubber stoppers and aluminum crimp seals. Separate bottles were used to quantify the N_2 produced in the batch cultures at the end of the incubation period. Batch cultures were evaluated with five concentrations of Cr^{VI} (0.1, 0.5, 1.0, 1.5 and 2 mg/L).

2.5 *Denitrifying batch cultures*

Batch experiments were undertaken in 125 mL serum bottles with basal medium (60 mL) as follows (mg/L): $NaNO_3$ (800), CH_3COONa (1000), $NaHCO_3$ (1500), K_2HPO_4 (4000), KH_2PO_4 (3500) and 1.5 mL/$L_{reactor}$ of trace elements solution (see 2.4 section). The experimental pH was of 7.5 ± 0.3. Batch cultures were spiked with 2 ± 0.02 g VSS/L. The liquid phase and headspace were bubbled for 6 min with He/CO_2 (80/20, v/v) in order to eliminate the oxygen from the bioassays. Separate bottles were used to quantify the N_2 produced in the batch cultures at the end of the incubation period. Batch cultures were evaluated with five concentrations of Cr^{VI} (0.1, 0.5, 1.0, 1.5 and 2 mg/L).

2.6 *Evaluation of the respiratory processes*

Microbial activity was evaluated in terms of consumption efficiencies (Eq. 1), production yields (Eq. 2) and specific consumption rates (Eq. 3). The kinetic parameters such as the specific rates were calculated using the Gompertz model (OriginPro 8.0). In all cases, the coefficient of determination (R^2) was higher than 0.96.

$$\%Ef = \frac{initial\ substrate - final\ substrate}{initial\ substrate} \times 100 \quad (1)$$

$$Y_{P/S} = \frac{mg\ of\ product}{mg\ of\ substrate\ consumed} \quad (2)$$

$$q = \frac{mg\ of\ substrate\ consumed}{g\ VSS\ h} \quad (3)$$

A modified non-competitive inhibition model was used to calculate the IC_{50} (Eq. 4). The IC_{50} is the chromium concentration that inhibits 50% of the microbial activity.

$$I\% = 100x\left[1 - \frac{1}{1 + ([Cr^{VI}/a])^b}\right] \quad (4)$$

where $I\%$ is the percentage of inhibition, Cr^{VI} is the inhibitor concentration (mg/L), "*a*" is the IC_{50} (mg/L), and "*b*" is the fitting parameter. Equation 4 was linearized to get the kinetic parameters (Jiang *et al.*, 2018).

2.7 *DNA extraction and Illumina Sequencing of 16S V4-V5 amplicons*

DNA extraction, integrity and size were made as was reported by Aguirre-Garrido *et al.*, 2016. After that, 5-20 ng of the sample was used for PCR amplification products of the V4 and V5 regions of the 16S rRNA gene through universal primers, 515 F and 926 R. The Nextera XT Index Kit v2 was used for the

amplicon multiplexing and sequencing (Illumina, San Diego, CA, USA). Illumina paired-end reads were used for analyzing the microbial sludge composition using the amplicons of the 16S rRNA gene through MiSeq instrument with 300+300 v3 kit chemistry at the University of CGEB-Dalhousie, Canada.

2.8 Bioinformatic analysis

The 16S rRNA sequences data were processed by using the MOTHUR software (Schloss *et al*., 2009; Kozich *et al*., 2013). Once demultiplexing with a minimum length of 400 bp and a maximum of 500 bp, chimeric reads were identified and excluded using the Chimera VSEARCH (Rognes *et al*., 2016). The bacterial composition of communities was determined by RDP Bayesian classifier Trainset 14, fix rank classification (Wang *et al*., 2007). The sequences that only could be classified at the lower classification level were used for further analysis.

2.9 Analytical methods

Ammonium nitrogen concentration was determined by a selective electrode, which detects the gas ammonia (Phoenix electrode company, USA). Soluble Chemical Oxygen Demand (COD) and volatile suspended solids (VSS) were determined according to the standard methods (APHA 2005). The chromium concentration was determined by UV spectrophotometer at 540 nm using 1,5-diphenylcarbazide (APHA 2005). Nitrite and nitrate pollutants were quantified by spectrophotometry at 300 and 350 nm, respectively (González-Blanco *et al*., 2017). The biogas production was measured using inverted columns with a saline solution (300 g NaCl/L, pH of 2). The biogas composition was determined at the end of the incubation period by gas chromatography (GOW-MAC Series 580). The kind of detector used for the chromatography was of thermal conductivity; with operating temperatures for the column, injector, and detector of 50, 100, and 110 °C, respectively.

3 Results and discussion

3.1 Control assays

In abiotic cultures spiked with Cr^{VI} plus ammonium, nitrate, nitrite and acetate either in presence or absence of oxygen, chemical reactions were not observed. Biotic assays with chromium were performed to verify sorption onto biomass. At the end of the incubation period, in all studies, almost all chromium concentration was recovered in the solution (approx. 98%); hence, sorption phenomena were discarded. For instance, Çeçen *et al*. (2010) suggested that due to the negative charge of chromium in solution (CrO_4^{2-}) could be discarded sorption phenomena since biomass surface usually has also negative charge. These experimental results suggested that the unique via followed for chromium removal was the biological reduction.

3.2 Biological cultures

Initially, batch cultures were performed in order to verify the activities of ANAMMOX, nitrification and denitrification of the microbial sludge obtained. Fig. 1A shows the time course of ANAMMOX process. Nitrite reduction was linked to ammonium consumption, achieving removal efficiencies of 81% ± 0.2 for nitrite, and 80% ± 0.1 for ammonium. The N_2 yield was of 0.98 ± 0.3 mg N_2 produced/ mg N- consumed. This yield value indicates that 98% of nitrogen consumed was recovery as N_2. The ammonium was oxidized at a specific rate of 0.67 ± 0.05 mg NH_4^+-N/gVSS h. For example, Gonzalez-Blanco *et al*. (2015) reported an ANAMMOX activity of 1.02 ± 0.05 mg NH_4^+-N/gVSS h in a denitrifying culture. The activity presented for the authors above mentioned was 1.5-fold faster than the ANAMMOX activity reported in the present study. This difference in the kinetic behavior might be associated that ANAMMOX bacteria in the natural wetland was in touch with chromium, diminishing their activity.

In Fig. 1B is shown the nitrate production profiles under nitrifying conditions. The ammonium removal efficiency was of 99.3% ± 1.2 with a nitrate production yield of 0.99 ± 0.01 mg NO_3^--N produced/ mg NH_4^+-N consumed. Nitrate was produced at a specific rate of 3.8 ± 0.2 mg NO_3^--N/ g VSS h (Table 1). For example, Velasco-Garduño *et al*. (2018) in an activated sludge system observed a nitrifying activity of 88 ± 3.2 mg NO_3^--N/g VSS d (approximately 3.66 mg NO_3^--N/g VSS h). The nitrifying activity reported by Velasco-Garduño *et al*. was similar to the nitrifying activity reported in the present work. These experimental results suggested that ANAMMOX activity was more sensible to the presence of chromium in the natural wetland than nitrifying activity. Finally, the microbial sludge taken from the rhizosphere showed the metabolic capability to carry out the nitrification and ANAMMOX processes.

Table 1. Substrate consumption efficiencies (%), NOx-N production yields (Y) and specific production and consumption rates in nitrifying cultures exposed to different concentrations of Cr^{VI}.

NH_4^+-N (mg/L)	Cr^{VI} (mg/L)	Y NO_3^--N/NH_4^+-N	Y NO_2^--N/NH_4^+-N	%E NH_4^+-N	*q_{NOx-N} (mg/gVSS h)	%E Cr^{VI}	q Cr^{VI} (mg/gVSS h)
75.2±0.8	control	0.99 ± 0.01	-	99.3 ± 1.2	3.8 ± 0.2	-	-
75.0±0.6	2.5 (**2.0) ± 0.02	1.0 ± 0.05	-	98.1 ± 1.8	3.1 ± 0.3	99.1 ± 1.1	0.07 ± 0.01
74.8±0.7	5.0 (4.0) ± 0.03	1.0 ± 0.02	-	88.8 ± 2.4	2.9 ± 0.2	73.3 ± 0.2	0.08 ± 0.01
76.2±0.5	10 (8.0) ± 0.08	0.96 ± 0.02	-	89.8 ± 2.0	2.6 ± 0.4	66.5 ± 0.2	0.023 ± 0.01
75±0.3	18 (16) ± 0.05	-	0.98±0.06	94 ± 0.2	4.8 ± 0.3	-	-
75±0.2	35 (32) ± 0.05	-	-	-	-	-	-

*q_{NOx-N}: production rate of nitrate or nitrite, ** Chromium in solution.

On the other hand, the microbial sludge did not show denitrifying activity in an incubation period of 72 h. Perhaps the biomass in touch with chromium in the natural wetland affected in some way the denitrifying metabolism. Interaction between Cr^{VI} and denitrifying bacteria in natural communities has not been well described, however, few studies indicate that denitrifying bacteria might be sensible to Cr^{VI}. For instance, Kourtev *et al.* (2009) showed that denitrifying activity was strongly inhibited by hexavalent chromium, nonetheless, Cr^{VI} did not eliminate the microbial community.

3.3 Effect of hexavalent chromium on nitrifying cultures

Fig. 1B shows the time course of nitrate production in presence of different chromium concentrations. At 2 mg Cr^{VI}/L, the ammonium removal efficiency was of 98.1% ± 1.8. Ammonium was fully oxidized over 40 h and it was recovered as nitrate, with nitrate yield of 1.0 ± 0.05 mg NO_3^--N produced/ NH_4^+-N consumed (Table 1). Nitrite as an intermediary was not observed. The kinetics of respiratory process of nitrification changed compared to the control study; since specific nitrate production rate diminished to 3.1 ± 0.3 mg NO_3^--N/ g VSS h, showing a decrease of 20%. Nitrifying cultures exposed to 4 and 8 mg Cr^{VI}/L showed ammonium removal efficiencies of 88.8% and 89.8%, respectively. Ammonium was completely oxidized to nitrate, with high nitrate yields; this means, that metabolism did not change. Nonetheless, hexavalent chromium showed a diminishment on the nitrifying kinetic, since specific nitrate production rates diminished to 2.9 and 2.6 mg NO_3^--N/ g VSS h, respectively (Table 1).

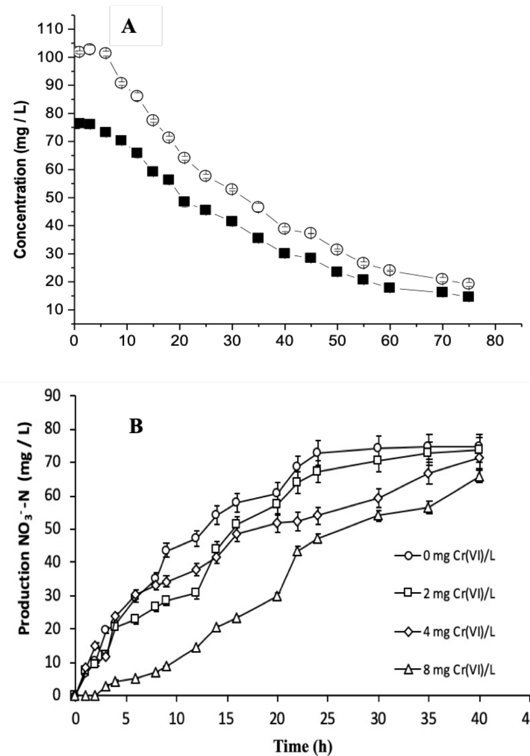

Fig. 1. **A)** Anammox activity in batch cultures (●) NO_2^--N; (■) NH_4^+-N, and B) Nitrate production profiles in presence of Cr^{VI} under nitrifying conditions.

In nitrifying cultures exposed to 16 mg/L, the kinetics and metabolism of the nitrification changed. Ammonium was partially oxidized over 35 h and it was recovered as nitrite, with nitrite yield of 0.98 ± 0.06 mg NO2–N/ NH_4^+-N consumed (Fig. 2A; Table 1). Nitrite was produced instead of nitrate, suggesting that chromium modified significantly the nitrifying metabolism.

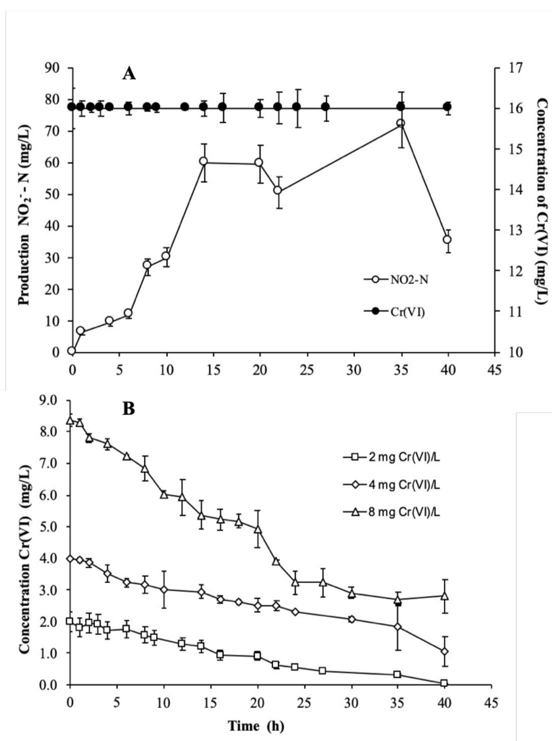

Fig. 2. **A)** Nitrite production profiles in presence of 16 mg Cr^{VI}/L under nitrifying conditions, and **B)** Chromium reduction profiles under nitrifying conditions.

The ammonium removal efficiency was of 94 ± 0.2%, whereas nitrite was produced at a specific rate of 4.8 ± 0.3 mg NO2–N/ g VSS h. Nitrate was not observed, suggesting that the nitrite-oxidizing enzyme was inhibited. The results contrast with Kim *et al*. (2016) who observed in a nitrifying continuous reactor fed with chromium, a greater diminishing of ammonium-oxidizing bacteria than nitrite-oxidizing bacteria, thus limiting nitrite as the substrate for nitrite oxidizing bacteria. At highest initial chromium concentration tested (32 mg Cr^{VI}/L), nitrifying activity was not observed, suggesting a strong inhibition (Table 1). The toxicity of Cr^{VI} has been associated with its capability to cross the cell membrane. Megharaj *et al*. (2003) and Kapoor *et al*. (2016) indicated that within the cell, Cr^{VI} is reduced to Cr^{III} producing complexes with cell components inhibiting the metabolic activity. Another possible mechanism of inhibition might be the competition between oxygen and hexavalent chromium as end electron acceptors (Wang and Xiao, 1995).

In Fig. 2B is shown the time course of chromium reduction under nitrifying conditions. At the initial concentration of 2 mg Cr^{VI} /L, the chromium removal efficiency was of 99.1% ± 1.1, while nitrifying cultures exposed to 4 mg/L and 8 mg/L of Cr^{VI}, the removal efficiencies diminished to 73.3% ± 0.2 and 66.5% ± 0.2, respectively (Table 1). These results show that under nitrifying conditions was possible to remove simultaneously chromium, nonetheless, the nitrification is the biological process more affected. For instance, Kapoor *et al*. (2016) and Su *et al*. (2016) observed that Cr^{VI} at a range of 1-5 mg/L inhibited strongly the nitrifying activity in batch cultures. In the present work, the IC_{50} value obtained for the nitrifying activity, in batch assays, was 7.9 mg Cr^{VI}/L. Vankova *et al*. (1999) studied the effect of hexavalent chromium on aerobic sludge activity and reported an IC_{50} value of 49 mg Cr^{VI} /L. The results indicated that nitrifying bacteria of the rizhosphere were more sensible to chromium than nitrifying bacteria of aerobic sludge systems reported by Vankova *et al*. (1999).

3.4 *Inhibition of ANAMMOX process by hexavalent chromium*

ANAMMOX cultures were not able to remove ammonium in the presence of chromium. Yu *et al*. (2016) showed that ANAMMOX bacterium has the enzymatic capability to reduce hexavalent chromium because it contains oxidoreductases and cytochromes. Even with these enzymatic characteristics, under the environmental conditions tested in the present study, Cr^{VI} reduction was not observed. Jiang *et al*. (2018) observed in a continuous-flow reactor that 1-2 mg Cr^{VI}/L stopped completely the ANAMMOX activity. In this context, ANAMMOX bacteria are very sensitive to the presence of chromium. Kosolapov *et al*. (2004) suggested that this type of bacteria usually perform only the absorption of metals in their cellular material such as ion exchange, chelation, adsorption or entrapment. For instance, Yu *et al*. (2016) reported an IC_{50} of 9.84 ± 0.61 mg Cr^{VI}/L for ANAMMOX cultures; the authors suggested that this tolerance might be attributed to the extracellular polymeric substances contributing to the attachment of heavy metals. In the present work, the initial Cr^{VI} concentration evaluated was below to the IC_{50} reported by Yu *et al*. (2016); nonetheless, the ANAMMOX activity completely ceased. Perhaps it might be due to the sort of sludge used in the present work since it was of type scattered. The scattered sludge has lower content of extracellular polymeric substances than flocculate or granular sludge.

Table 2. Substrate consumption efficiencies (%), N_2 production yields and lag phases (λ) of ANAMMOX cultures exposed to different concentrations of Cr^{VI}.

Sludge exposed to Cr^{VI} (mg /L)	%E NO_2^--N	%E NH_4^+-N	$Y_{N_2/N\text{-consumed}}$	λ (h)
Control	81.0 ± 2.0	80.0 ± 0.1	0.98 ± 0.03	2
0.1 ±0. 01	72.7 ± 0.8	70.9 ± 1.1	0.97 ± 0.04	120
0.5 ± 0.01	59.8 ± 1.1	55.8 ± 0.4	0.98 ± 0.05	134
1.0 ± 0.02	32.7 ± 1.4	31.0 ± 1.3	0.98 ± 0.04	152
1.5 ± 0.01	15.1 ± 1.2	12.6 ± 0.8	0.92 ± 0.03	160
2.0 ± 0.01	11.2 ± 0.7	8.45 ± 1.1	NA	176

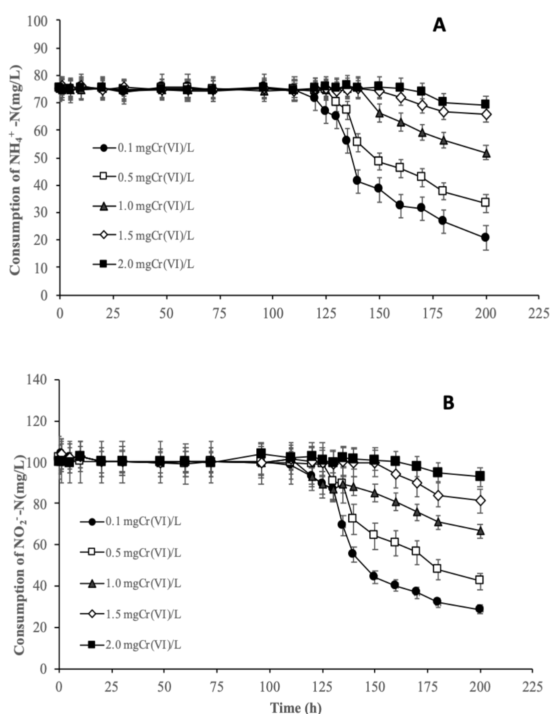

Fig. 3. Recovery of ANAMMOX cultures. **(A)** Ammonium consumption profiles, **(B)** Nitrite consumption profiles.

3.5 Recovery cultures

In this section, microbial sludge exposed to chromium in the ANAMMOX cultures (section 3.4) was used to evaluate its recovery capability under optimal ANAMMOX conditions, without chromium, for an incubation period of 200 h (Fig. 3). In ANAMMOX cultures where microbial sludge was exposed to 0.1 mg Cr^{VI}/L, lag phase was observed, being of 120 h (Table 2). After the lag phase, concomitant ammonium oxidation together nitrite reduction was observed. Nitrite and ammonium removal efficiencies attained were 72.7% and 70.9%, respectively (Table 2). N_2 was the end product, with a yield of 0.97 ± 0.04 mg N_2/ mg N- consumed. In batch cultures where sludge was exposed to 0.5 mg Cr^{VI}/L, the lag phase increased to 134 h. The removal efficiencies for nitrite and ammonium were 59.8% and 55.8%, respectively, with N_2 yield of 0.98 ± 0.05 mg N_2/ mg N- consumed. In batch cultures where biomass was exposed to 1.0 and 1.5 mg Cr^{VI}/L, the lag phase increased to 152 h and 160 h, respectively. At the end of the incubation period, the removal efficiencies for nitrite and ammonium were 32.7% and 31% for 1.0 mg Cr^{VI}/L, and 15.1% and 12.6% for 1.5 mg Cr^{VI}/L, respectively. The end product was N_2, with yields of 0.98 ± 0.04 and 0.92 ± 0.03 mg N_2/ mg N- consumed, respectively.

Finally, in batch cultures where microbial sludge was exposed to the highest chromium concentration, the lag phase increased up to 176 h, and N_2 production was not observed. At this last chromium concentration tested, the substrates removal efficiencies were the lowest obtained. In all batch cultures, the lag phase (λ) was observed; this means that the adaptation phase took almost 5-7.5 days for the substrate consumption. Hence, after adaptation phase ammonium and nitrite started to be consumed, suggesting reversible inhibition phenomenon. The results indicated that microbial sludge exposed to chromium was affected in some way since it required enough time for recovering ANAMMOX activity. For instance, Vaiopoulou and Gikas (2012) suggested that chromium causes enzymatic activity damage, which would require time and suitable environmental circumstances to be repaired.

3.6 Microbial community structure

Fig. 4 shows the relative abundance of the microbial sludge used for the biological studies. *Xanthomonadaceae* (17.17%), *Ignavibacteriaceae*

(16.52%), *Trueperaceae* (10.66%), *Chitinophagaceae* (10.06%) and *Nitrosomonas* (7.07%) were relatively abundant. Fitzgerald *et al*. (2015) observed that *Pseudomonas, Xanthomonadaceae, Rhodococcus*, and *Sphingomonas* were involved on nitrification under low dissolved oxygen conditions. Connan *et al*. (2017) identified *Ignavibacteriaceae* family on ANAMMOX cultures; the authors suggested that those bacteria use organic matter coming from other cells. *Nitrosomonas* are nitrifying bacteria with the metabolic capability to oxidize ammonium (Keluskar *et al*., 2013). The family *Trueperaceae* can grow under extreme conditions, such as in moderate saline and alkaline habitats, as well as low temperatures (Ivanova *et al*., 2011). Li *et al*. (2018) observed in a bio-electrocoagulation technology for nitrogen removal at low temperatures that the relative abundance of *Trueperaceae* increased. For example, *Truepera* belongs to family *Trueperaceae*, which can degrade organic acids, polyols, carbohydrates and amino acids (Albuquerque *et al*., 2005). On the other hand, Bartelme *et al*. (2017) in a nitrifying biofilter expected a predominance of nitrifying bacteria; nonetheless, the results were contrary since they observed that *Chitinophagaceae* (~12%) and *Acidobacteria* (~9%) dominated, whereas *Nitrosomonas* and *Nitrobacter* were in lesser proportion. On the other hand, *Xanthomonadaceae, Ignavibacteriaceae, Trueperaceae* and *Chitinophagaceae* are often detected in nitrogen removal systems, so identifying the roles and interactions of such bacteria inside nitrogen removal reactors is a great challenge for upcoming studies.

In the microbial sludge, *Planctomycetes* were also identified, but the relative abundance was less than 2%. All known ANAMMOX bacteria species belong to Planctomycetes phylum such as *Ca*. Brocadia, *Ca*. Kuenenia, *Ca*. Scalindua, *Ca*. Anammoxoglobu, *Ca*. Jettenia, and *Ca*. Anammoximicrobium (Pereira *et al*., 2017). In the present work, despite the low relative abundance of *Planctomycetes*, the ANAMMOX activity in the microbial sludge was observed. On the other hand, *Thiobacillus, Comamonadaceae*, and *Castellaniella* are denitrifying bacteria, and their relative abundance in the microbial sludge was low. The members of the family *Thiobacillus* and *Comamonadaceae* are hydrogenotrophic denitrifying bacteria (Park *et al*., 2016).

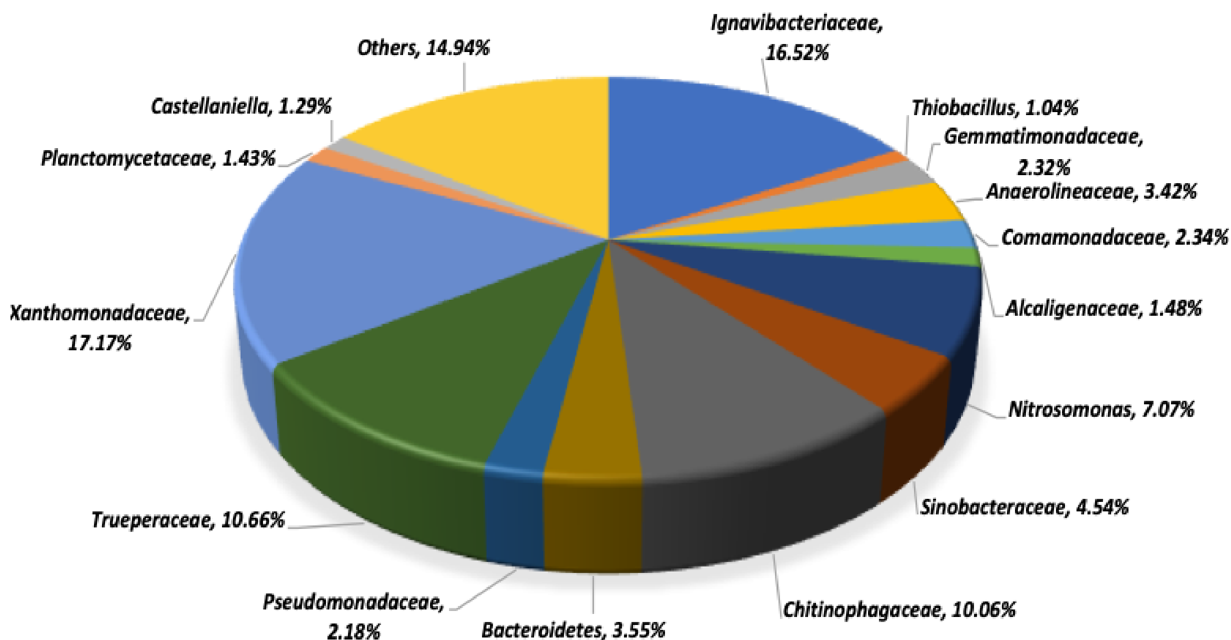

Fig. 4. The percentage distribution of the total bacterial community.

The genus *Castellaniella* was initially described by Kämpfer *et al*. (2006) to be facultative and denitrifying. The genus *Castellaniella* consists of two species, *C. defragrans* and *C. denitrificans* (Spain *et al*., 2007), being the last a phenol-degrading denitrifying bacterium (Baek *et al*., 2003). In the present work, the sludge of the rhizosphere was not able to carry out the denitrification, perhaps due to the prolonged contact of denitrifying bacteria and chromium in the natural wetland. The denitrifying bacteria are not tolerant to the presence of this metal, as was reported by Kourtev *et al*. (2009).

Conclusions

The microbial sludge taken from the natural wetland polluted with chromium was able to nitrify and carry out the ANAMMOX, however denitrifying activity was not observed. Hexavalent chromium inhibited the nitrifying activity, whereas the respiratory process of ANAMMOX was fully stopped. Recovery cultures showed that ANAMMOX bacteria suffered some damage by chromium presence since they required more than 5 days to recover the biological activity. The microbial composition of the sludge was shaped mainly by *Xanthomonadaceae, Ignavibacteriaceae, Trueperaceae*, and *Chitinophagaceae*, followed by *Nitrosomonas, Planctomycetes, Comamonadaceae, Castellaniella*, and *Thiobacillus*.

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