Acclimation of nitrifying biomass and its effect on 2-chlorophenol removal

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ABSTRACT

The metabolic and kinetic behavior of a nitrifying sludge exposed to 2-chlorophenol (2-CP) was evaluated in batch cultures. Two kinds of nitrifying culture were used; one acclimated to 4-methylphenol (4-mp), and the other unacclimated to 4-mp. The unacclimated culture was affected adversely by the 2-CP's presence, since neither nitrification nor 2-CP oxidation was observed. Nonetheless, the acclimated culture showed metabolic capacity to nitrify and mineralize 2-CP. Ammonium removal was 100%, with a nitrifying yield of 0.92 ± 0.04 mg NO_3-N/mg NH_4^+-N consumed. The consumption efficiency for 2-CP was 100% and the halogenated compound was mineralized to CO_2. Denaturing gradient gel electrophoresis (DGGE) patterns showed the shift in microbial community structure, indicating that microbial diversity was due to the acclimation process. This is the first evidence where nitrifying culture acclimated to 4-mp completely removed ammonium and 2-CP.

INTRODUCTION

2-Chlorophenol (2-CP) is a persistent contaminant that occurs during anthropogenic activities and is released to different ecosystems, such as water, air and soil (Vidal & Diez 2003). Wastewater from industries such as polymeric resin production, oil refining, petrochemicals, paper and coking plants also contain this kind of CP (Quan et al.2003). This compound is considered to be a serious pollutant because of its toxicity and significant environmental impact.

Biodegradation of CP by microorganisms is more specific, environmental friendly and relatively inexpensive (Eker & Kargi 2006). Several biological processes have been tested in order to remove CP from wastewater either in anaerobic or aerobic conditions (Bali & Sengu 2003 Zilouei et al. 2006; Martinez-Hernández et al. 2011; Silva et al. 2011). For example, Satoh et al. (2005) studied the effect of 2-CP on a nitrifying biofilm, observing a decrease in the ammonium consumption rate, but 2-CP was not con-sumed. Majumder et al. (2007) tested a similar concentration of 2-CP using an aerobic rotating biological contact, but this pollutant was consumed after 215 days of acclimation. Martinez-Hernández et al. (2011) studied the effect of 5 mg/L of 2-CP on nitrifying sludge in batch cultures, observing ammonium removal of 10%, and that the nitrifying sludge required a long time (around 30 days) for 2-CP depletion. Silva et al. (2011) worked with aerobic cultures acclimated to a mixture of phenolic compounds, observing phenolics and ammonium removal. Nonetheless, studies on the effect of CP on nitrification are still scarce in the literature.

Aerobic granular sludges from domestic sewage treat-ment plants are frequently used as inoculum to start-up the biological treatment process. Thus, it appeared useful to investigate the process of acclimation for 2-CP removal with-out losing the nitrification. The procedure of sludge acclimation would be developed for the plant operators, in order to shorten the start-up and to provide an efficient pro-cess. Therefore, the objective of this work was to determine the effects of 2-CP on the nitrifying sludge acclimated and unacclimated to 4-mp using response variables such as specific rates and product yields as well as to obtain evi-dence of the level to which this compound acts. In the present study denaturing gradient gel electrophoresis (DGGE) was chosen as the fingerprinting method to provide a rapid visual indication of variations in microbial commu-nity structure.

Key words | 2-chlorophenol, acclimation, DGGE-pattern, nitrification

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MATERIALS AND METHODS

Inoculum source

Nitrifying biomass cultivated in a continuous-flow reactor (R_{-1}) for more than 1 year was used for inoculating a second continuous stirred tank reactor (R_{-2}), which was operated for 6 months. R_{-1} was fed with ammonium (unacclimated culture), and R_{-2} with ammonium and 4-mp (acclimated culture). Bioreactors were continuously aerated and operated at 25 °C, pH 7.6 ± 0.3 with a hydrau-lic retention time of 2 days. The reactors were fed with mineral media divided into two tanks, (A) containing nitrogen source and (B) containing carbon source as follows (g/L), Medium A: $(NH_4)_2SO_4$ (1.73), NH_4Cl (1.4), KH_2PO_4 (2.73), $MgSO_4$ (0.6), NaCl (1); and Medium B: $NaHCO_3$ (9.3) and $CaCl_2$ (0.04). The sludge was washed with a solution of NaCl (9 g/L) and centri-fuged (4,000 g, 10 minutes) before being used in the batch assays.

Batch bioassays

Batch assays were undertaken in serological bottles of 160 mL. Sludge incubations contained 100 mL of basal medium and were inoculated with the previously stabil-ized nitrifying sludge taken from R_{-1} and R_{-2} (approximately 208 mg microbial protein/L). The culture medium consisted of (g/L): $(NH_4)_2SO4$ (0.24), NH_4Cl (0.19), KH_2PO_4 (0.028), $MgSO_4$ (0.02), NaCl (0.02), $NaHCO_3$ (1.75) and $CaCl_2$ (0.01). The pH of the basal medium was controlled at 7.5 ± 0.3 by the addition of bicarbonate, which was corroborated by pH determi-nations during the incubation period. The headspace and liquid were flushed with pure oxygen for 4 minutes in order to provide oxygenated cultures. All experimental treatments were carried out in duplicate and incubated at 30 °C in a shaker at 200 rpm. Each incubation bottle was an independent experimental unit, which was sacrificed after sampling. The response variables used to evaluate the behavior of the nitrifying sludge were consumption efficiencies ($E_{NH_4^+}$ or E_{p-Cr}, (mg substrate consumed/mg substrate fed) × 100), nitrate production yields (Y_{NO_3}, mg NO_3^--N/mg substrate consumed), and specific consump-

tion rates of NH_4^+-N ($q_{NH_4^+}$), and NO_3^--N (q_{NO_3}). Average specific rates (q, mg substrate or product/g protein h) and lag phases (λ) were calculated by using the Gompertz model, as was described by Beristain-Cardoso *et al.* (2009).

Microbiological analysis

The DNA was obtained from the continuous stirred tank reac-tors; extraction was performed with an AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA). The polymerase chain reaction (PCR) was performed using the primers Bac-968f and Bac-1401r (UNAM, Instituto de Biotecnología, México DF, Mexico) designed for van Leerdam *et al.* (2000) by attaching a tail of 40 bp of G and C at the 5′ primer forward. We used 3 mM MgCl, 0.2 mM dNTPs, 0.4 μM each of the primers, 80 ng DNA and 1 unit of enzyme brand Vent DNA polymerase (New England BioLabs, Wilbury, Hitchin, UK). The reaction was subjected to the next program in the thermocycler at 94 °C for 5-minute denaturation, then 30 cycles of amplifica-tion, using 94 °C for 30 seconds for denaturation, 54 °C for 2 minutes for annealing and 72 °C for 1 minute for polymeriz-ation. The PCR products were run in an acrylamide gel denaturing gradient using team D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). Ten micrograms were placed in each of the PCR products on a gel containing 6% acrylamide denaturing gradi-ent of 48–52% of urea and formamide at 60 °C with an initial voltage of 200 V for 10 minutes, and then 85 V for 16 hours using TAE running buffer to 0.5×. After the shift, the gel was stained with silver nitrate according to the protocol of Sanguinetty *et al.* (1994).

Analytical methods

Ammonium was analyzed by a selective electrode (Phoenix Electrode Company, Houston, TX, USA). Nitrite and nitrate were analyzed by high-performance liquid chromatography (HPLC) (PerkinElmer series 200 HPLC Systems, Shelton, CT), using an ion exchange column (IC-Pak Anion HC, 4.6 × 150 mm, Waters, Dionex Corporation, Sunnyvale, CA, USA) and a UV detector at 214 nm; 2-CP was quantified by HPLC (PerkinElmer, HPLC Systems, Shelton, CT, USA), using a C-18 reverse phase column (Phenomenex, Torrance, CA, USA) and a UV detector at 274 nm, being the mobile phase acetonitrile/water (60:40 v/v). Total organic carbon was measured by TOC analyzer (TOC-5000, Shimadzu, Kyoto, Japan). Microbial protein was determined by Lowry's method (Lowry *et al.* 1951), and pH and dissolved oxygen were determined using specific electrodes. Scanning electron microscopy (SEM) was used to observe the micro-organisms inside the aerobic granules, according to Bozzola & Russell (1991).

RESULTS AND DISCUSSION

Reactor performance

Table 1 shows the response variables of both reactors at steady-state. In R_1, $E_{NH_4^+}$ was 100%, with Y_{NO_3} 0.96 ± 0.02. In R_2, $E_{NH_4^+}$ and E_{p-Cr} were 100% ± 4.0 and 95% ± 5.0, respectively, with Y_{NO_3} 0.95 ± 0.03. Soluble organic carbon in the output was not detected, suggesting that the *p*-cresol consumed was mainly mineralized. At steady-state conditions, both kinds of sludge were taken from the reac-tors in order to evaluate the acclimation process for simultaneous removal of ammonium and 2-CP.

Nitrifying activity as control assays

Nitrifying profiles with unacclimated and acclimated sludge are shown in Figures 1 and 2. The unacclimated culture consumed NH_4^+-N in 7.5 hours (Figure 1). A concomitant formation of NO_3^- with a transitory NO_2^- formation was observed. Figure 1 shows that nitrite consumption was made within 8 hours. After 8 hours, for both studies, the substrate consumption efficiencies and nitrate yields were very high (Table 2). The unacclimated culture removed ammonium with $q_{NH_4^+}$ of 193 ± 8.0 mg NH_4^+-N/ g protein h, while the q_{NO_3} was 94 ± 7.0 NO_3^--N/ g protein h. The acclimated culture displayed a similar metabolic behavior to unacclimated culture, since $E_{NH_4^+}$ and Y_{NO_3} did not significantly change (Figure 2). However, $q_{NH_4^+}$ and q_{NO_3} diminished at 45% and 27%, respectively, in compari-son with the unacclimated culture. These experimental results suggested that the biomass exposed to 4-mp did not affect the nitrifying metabolism. But the kinetic aspect was altered, displaying a diminishment on specific rates. Bio-mass exposed to 4-mp in continuous-flow culture might have induced changes in chemical structure at membrane level; that is, isomerization of *cis*-unsaturated fatty acids to the *trans*-configuration as was reported by Heipieper *et al.*(12) and van Schie & Young (2000). The modification of the chemical structure of the membrane might have altered the substrate transport.

Nitrifying activity in the presence of 2-CP

The effect of 2-CP on nitrifying activity with unacclimated and acclimated sludge is shown in Figures 3 and 4. The unacclimated culture was not able to nitrify in the presence of 2-CP, and the phenolic compound was not consumed in the evaluated period. Satoh *et al.* (5200) evaluated the effect of 10 mg/L of 2-CP on a nitrifying biofilm not exposed to phenolic compounds, finding that nitrifying consumption rates were inhibited, and 2-CP was not consumed. Martínez-Hernández *et al.* (2011) worked with biomass not acclimated to phenolic compounds, showing that nitrifying activity was strongly affected by 5 mg/L of 2-CP, and halogenated com-pound consumption took 30 days.

In the present work, the acclimated culture was able to nitrify and to oxidize 2-CP (Figures 3 and 4). The culture consumed ammonium in 120 hours (~3 days). A concomi-tant formation of NO_3^- with a transitory NO_2^- formation was observed, and nitrite consumption was made within 120 hours. The $E_{NH_4^+}$ was 100%, with Y_{NO_3} 0.92 ± 0.04. Ammonium was consumed at a specific rate of 6.3 ± 1.0 mg NH_4^+-N/ g protein h, after a lag phase of 12 hours (Table 2). 2-CP consumption started around 75 hours, with a pronounced linear consumption. 2-CP was oxidized at a specific rate of 0.36 ± 0.03 mg C/ g protein h, with

nitrifying controls (Table 2). These experimental results showed that the presence of 2-CP diminished the specific rates of nitrification; nonetheless, high removal efficiencies for ammonium and 2-CP were obtained. Martínez-Hernán-dez *et al.* (2011) reported a consumption of 5 mg/L of 2-CP in 30 days, whereas in the present work, 5 mg/L of 2-CP was removed in 2.5 days by using acclimated sludge. In the literature, there are microorganisms with an intrinsic tolerance to resist highly-toxic compound concentrations; this intrinsic property can be defined as genetic properties that allow cells to avoid biocidal action (McLellan *et al.*7200). However, gram-negative bacteria such as *Pseudomo-nas* and *Vibrio* have presented the isomerization of the fatty acids *cis* to *trans* position (Heipieper *et al.* 1992; Weber *et al.* 1994), which seems to be a system of adaptation to high concentrations of toxicity. In the present work, the acclimation process was crucial for 2-CP removal without losing nitrification, and this information should be taken as a sludge acclimation procedure for plant operators, par-ticularly to treat wastewater polluted with nitrogen and halogenated compounds.

Analysis of DGGE pattern

The compositions of bacterial communities from the reac-tors in steady-state conditions were compared using nested PCR-DGGE and the amplified fragments were derived from the ribosomal 16S region. Differences among lanes in both DGGE profiles of bacterial communities were clearly observed (Figure 5). There are many visible bands showing diversity of microbial ecology in the sludge samples. During R_{-1} operation at steady-state conditions, seven bands with high intensity were identified. It is reason-able to suppose that those bands derive from nitrifiers, since the reactor was operated under strict nitrifying conditions for more than 1 year. In the DGGE column, for R_{-2}, six bands with high intensity were identified. Three of them (marked with dashed arrows) remained, so that being 4-mp fed did not affect these microbial populations. This be-havior might be attributed to the following reasons: (1) acclimation at membrane level, as described above, or (2) perhaps these populations had the enzymatic capacity to oxidize 4-mp. For example, Radniecki *et al.* (2008) showed changes at membrane level after the exposition of *Nitroso-monas europae* to toluene and benzene. On the other hand, nitrifiers have been reported where ammonium mono-oxygenase (AMO) enzyme is able to oxidize either organic compounds or ammonium (McCarty 1999).

In R_{-2}, new bands were displayed (A-1, A-2, and A-3). This information clearly shows that 4-mp addition affected the sludge's microbial community by causing the prolifer-ation of heterotrophic bacteria owing to the heterotrophic conditions. Microbial populations involved in phenolic com-pound removal might be heterotrophic aerobic bacteria or heterotrophic nitrifiers. For instance, Moir *et al.* (1996) reported that the heterotroph (i.e., *Paracoccus*) also contains the AMO enzyme, thus contributing to the organic matter and to ammonium oxidation.

When nitrifying sludge from R_{-1} was exposed to 2-CP, nitri-fying activity was lost, suggesting that 2-CP exerted a toxic effect on these microbial populations. Kenner & Arp (1994) reported that ammonium oxidation is the step most affected when aro-matic compounds, such as benzene, alkyl benzenes, phenol, acetophenone, pyridine and benzoic acid, are present in the culture medium. However, in the present work, the same nitri-fying sludge (but acclimated to 4-mp) removed 2-CP and ammonium. The successful removal of both compounds might have been associated with the microflora diversity owing to the acclimation process. The microbial diversity was also confirmed by SEM images (Figure 6). In granular sludge not exposed to phenolic compounds, the predominant mor-phology was short straight rods with sizes ranging from 0.5 to 1.0 μm long (Figure 6(a)). However, the predominant mor-phology inside the aerobic granular sludge exposed to *p*-cresol was filamentous bacteria comprising long chains of small bacterial cells with sizes about 10 μm long (Figure 6(b)).

Finally, nitrifying sludge previously exposed to 4-mp pre-served the nitrifying capacity and it also showed the metabolic capacity to mineralize 2-CP; this metabolic behavior might indicate that the sludge in contact with 4-mp acquired the ability to tolerate and consume similar compounds, and the presence of 4-mp also promoted the proliferation of micro-flora involved in organic matter consumption.

CONCLUSIONS

These experimental results showed that the acclimation pro-cess was crucial for removing 2-CP without losing the nitrification. Certain culture conditions, bacterial popu-lations or communities vary, which was essential for 2-CP removal. DGGE allowed the determination of shift in microbial populations, facilitating the understanding of population dynamics. This scientific information is quite rel-evant for treating wastewater polluted with nitrogen and halogenated compounds.

ACKNOWLEDGEMENTS

This project was supported by CONACyT, México (CB-2011-01 164746) and Universidad Autónoma de la Ciudad de México (UACM/OAG/ADI/006/2011) (UACM/OAG/ADI/006/2011).

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