Concurrence of Anaerobic Ammonium Oxidation and Organotrophic Denitrification in Presence of *p*-Cresol

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Abstract

This study was carried out to evaluate the capacity of anaerobic granular sludge for oxidizing ammonium and *p* cresol with nitrate as terminal electron acceptor. Kinetics for the anaerobic oxidation of ammonium and *p* cresol is de scribed in this paper. The phenolic compound was very efficiently consumed, achieving 65 % of mineralization. Ammonium and nitrate were also consumed at 83 and 92 %, respectively, being the main product N_2.

Anaerobic ammonium oxidation was promoted owing to accumulation of nitrite, and it allowed the synergy of anaerobic ammonium oxidation and organotrophic denitrification for the simulta neous removal of ammonium, nitrate, and *p* cresol. A carbonaceous intermediate partially identified was transiently accumulated, and it transitorily truncated the respiratory process of denitrification. These experimental results might be considered for defining strategies in order to remove nitrate, ammonium, and phenolic com pounds from wastewaters.

Keywords Denitrification . Anaerobic ammonium oxidation . Nitrite . p-Cresol . Intermediates

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Introduction

Cresols and nitrogenous compounds (i.e., ammonia, nitrate) have been discharged into aquatic ecosystems, such as surface or groundwaters, through their use as biocides, fertilizers or generated by the petrochemical or chemical industry [1, 2]. For instance, in sour water from petrochemical industry is common to find *p* cresol, ammonium, and nitrate [3]. The US Environmental Protection Agency has classified cresols as pollutant of group C (possible human carcinogen) [4]. *p* Cresol, even at very low concentration, has adverse effects on the central nervous system, cardiovascular system, lungs, and kidneys and can cause central nervous system depression [5]. On the other hand, ammonia is one of the main nitrogenous compounds that can lead to eutrophication, hypoxia, and loss of biodiversity and habitat in water bodies [6, 7].

Biological removal of these compounds is more environmentally friendly than physico chemical processes [8]. Ammonium is usually oxidized (to nitrate) and then reduced to N_2 by sequential nitrification denitrification or directly through anaerobic oxidation (anammox). The first step in nitrification denitrification is the aerobic oxidation of ammonium into nitrate. The second stage is denitrification, where nitrate is reduced to N_2. Denitrifying microorganisms use nitrate or nitrite as the final electron acceptor [9, 10]. In this step, organic or inorganic compounds are needed as electron donors. Denitrifying sludge has the versatility to oxidize an extensive group of organic compounds, such as methanol, acetate, propionate, ethanol, phenol, and *p* cresol. [11]. Likewise, denitrifying consortia can use inorganic energy sources, such as sulfide, which is oxidized to sulfate with elemental sulfur as a possible intermediate product [12, 13]. N_2 production from anaerobic ammonia oxidation via anammox is carried out only when nitrite is present, but not with nitrate [14 16]. The presence of organic matter has been seen as inhibitory to this autotrophic process [17, 18]. Therefore, wastewaters containing low levels of organic carbon and high nitrogen concentrations might be treated via anammox process. However, some studies have documented that the coupling between anammox and organotrophic denitrification can contribute to the simultaneous oxidation of ammonium and organic substrates, including phenolic compounds, linked to the reduction of nitrite/nitrate [19, 20]. For instance, Cervantes et al. [21] evaluated several ammonium loading rates (25 500 mg NH_4^+/L day) in a denitrifying continuous UASB reactor using nitrate as electron acceptor. N_2 production rates increased, whereas ammonium loading rates were increasing. The authors suggested that overproduction of N_2 might have been associated to the coupling between anammox and organotrophic denitrification processes.

It has been observed that nitrite can be accumulated during denitrification when the electron donor is stoichiometrically insufficient, when nitrate reduction proceeds faster than nitrite reduction, or when the nitrite reductase enzyme is inhibited [22, 18]. Meza et al. [23] observed transient nitrite accumulation when *p* cresol was added as reducing source during denitrifica tion. In some cases, this accumulation might negatively affect the respiratory process. How ever, the promotion of nitrite accumulation might also be a good alternative to achieve anaerobic ammonium oxidation into molecular nitrogen, with the concomitant mineralization of phenolic compounds under denitrifying conditions. Nevertheless, a better understanding about the metabolism and kinetic behavior of the denitrifying sludge is still necessary to overcome this challenge. The aim of this work was to evaluate the metabolic capability of denitrifying sludge to achieve simultaneous removal of ammonium, nitrate, and *p* cresol, with the overall goal of obtaining N_2 and CO_2 as the main products. This research might be crucial because industrial wastewaters are highly heterogeneous and several of them are polluted with these compounds.

Materials and Methods

Biomass Stabilization in a Denitrifying Reactor

A 5 L completely stirred tank reactor (CSTR) was operated at 30±0.2 °C with a hydraulic retention time of 3.2 days for 2 years. The reactor was inoculated with 4 g volatile suspended solids (VSS)/L taken from a UASB reactor installed at Metropolitan Autonomous University Iztapalapa, Mexico. The CSTR was fed at the following loading rates (mg/L day): 128 of NO_3 N, 258 of acetate C and 22 of NH_4^+ N. The basal mineral medium was composed of (g/L) K_2HPO_4 (4.5), KH_2PO_4 (3.0), and trace elements solution supplied at 1.5 mL/L. The trace element solution contained (g/L) $CaCl_2{\cdot}2H_2O$ (5.54), $MnCl_2$ (5.0), $MgCl_2$ (5.0), EDTA (5), $CoCl_2{\cdot}6H_2O$ (1.6), $CuSO_4{\cdot}5H_2O$ (1.57), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (1.1), and $FeCl_3{\cdot}6H_2O$ (0.05). Nitrate, nitrite, ammonium, acetate, and bicarbonate were quantified in the denitrifying reactor. Biogas production and composition were also regularly monitored in the reactor. Table 1 summarizes the reactor performance under steady state conditions. The consumption efficiencies of nitrate, acetate, and ammonium were of 98.4, 76.4, and 88.7 %, respectively, being N_2 and HCO_3 the main end products. The product yield of molecular nitrogen (YN_2) was of 0.91 mg of N_2/mg of NO_3 N fed, and the bicarbonate yield ($YHCO_3$) of 0.62 mg of total inorganic carbon/mg of acetate C fed. Batch cultures were carried out using the sludge produced in the CSTR at steady state.

Batch Assays

Batch experiments were undertaken in serologic bottles of 160 mL. Sludge incubations contained 60 mL of basal medium with the composition described above. The serum bottles were spiked with 35.5±0.37 mg*p* cresol C(*p* Cr)/L, 93.3±2.1 mg NO_3 N/L, and 63.7± 1.7 mg NH_4^+ N/L. The C/N ratio was around 0.38 mg C/mg N, according to Eq. 1.

$$C_7H_8O + 17\,NO_3 + 3H_2O \rightarrow 7\,HCO_3 + 7\,H^+ + 17\,NO_2 \quad (1)$$

The batch assays were inoculated with 1.9±0.03 g VSS/L of denitrifying sludge from the CSTR. The headspace was flushed for 10 min with helium to establish anaerobic conditions. All bottles were sealed with butyl rubber stoppers and aluminum crimp seals. Separate bottles were set up for N_2 measurements. In order to discard chemical reactions, abiotic controls lacking inoculum were run at the same initial concentrations regarding denitrifying cultures. Biotic controls individually amended with only NH4+-N, NO3−-N, or p-Cr were also included. All assays were carried out by triplicate, and every incubation bottle was an independent experimental unit, which was sacrificed after sampling. The bottles were incubated at 30 °C, pH of 7.1±0.5, and 200 rpm of agitation in an orbital shaker. Microbial performance was evaluated in terms of consumption efficiency [E, %, mg of C or N consumed/mg of C or N fed]×100, yield [Y,mgofC or N produced/mg of C or N consumed], specific substrate consumption rate [qS,mgofCor N consumed/g VSS h] and specific production rate [qP, mg of C or N produced/g VSS h]. The specific rates were calculated by the integrated Gompertz model [20]. The coefficient of deter-mination (R2) was higher than 0.9 for all respiratory rates calculated.

Analytical Methods

NO_3 and NO_2 were analyzed by capillary electrophoresis (Beckman Coulter, proteomeLab PA 800), and buffer was prepared with 5 mL of Na_2SO_4 (0.1 M), NaCl (10 mM), and a commercial solution CIA Pak OFM anion BT (Waters) plus 35 mL of deionized water. A microcapillar of melted silica (60 cm long and 75 mm internal diameter) was used and a UV detector at 254 nm. N_2, CO_2, and N_2O were measured by gas chromatography with thermal conductivity detector. Temperatures for the column, injector, and detector were 50, 100, and 110 °C, respectively. Helium was used as carrier gas at a constant flow rate of 16 mL/min. The stainless steel column (Porapak Q mesh 100 80) was of 1.20 m long and 1/8″ diameter. The NH_4^+ was measured using an ion selective electrode (pHoenix electrode Co. Mod. NH331501). *p* Cr, *p* hydroxy benzylalcohol C (*p*OHBzalc), *p* hydroxy benzoate C (*p*OHBzate), and *p* hydroxy benzaldehyde C (*p*OHBzald) were determined by high performance liquid chromatography (HPLC) (PerkinElmer series 200 UV) using a C18 reverse phase column and a UV detector at 280 nm. The mobile phase was an acetonitrile:water (60:40 *v*/*v*) mixture at 1.5 mL/min. Scanning was performed by using high performance liquid chromatography (Waters, Milford, MA, USA) equipped with diode array detector model 2996, at a wavelength of 190 to 350 nm. Mobile phase, flow, and column type were the same than for the measurement of phenolic compounds. Inorganic carbon was measured in a TOC meter (Shimadzu Co. Model TOC 5000A). Before sampling, all liquid samples were filtered through a 0.45 μm nylon membrane. All the other measurements (pH and VSS) were according to standard methods [24].

Results and Discussion

Control Assays

Batch assays were performed for a period of approximately 90 h. Abiotic cultures spiked with NO_3 N, NH_4^+ N, and *p* Cr did not show any chemical reaction, because at the end of the batch experiments the initial concentrations remain without change. Regarding inoculated treatments, *p* Cr and NH_4^+ N consumptions without electron acceptor (NO_3 N) and vice versa were evaluated in order to observe a contribution of a possible endogenous metabolism. At the end of the batch assays nitrate, ammonium and *p* Cr were almost completely recovered (~94 %). These experimental data evidenced that consumption of three substrates was mainly associated to denitrifying sludge activity.

Batch controls with only nitrite and ammonium were performed in order to evaluate the sludge's capacity to ammonium oxidation. Nitrite and ammonium were consumed simultaneously, being the main product molecular nitrogen (Supplementary Fig. 1). After 30 h, nitrate started to be produced and it is linked to the anabolism process as has been observed in the anammox process.

Nitrate Reduction

Batch assays were carried out in order to evaluate the metabolic behavior of anaerobic sludge in the presence of two nitrogenous compounds, NO_3 and NH_4^+, and one phenolic compound,
p cresol, namely, one terminal electron acceptor and two energy sources, respectively. Figure 1a shows the time course of nitrogen compounds consumption. In global terms, nitrate was readily consumed at specific rate of 0.80±0.11 mg NO_3 N/g VSS h, achieving con sumption efficiency of 92 % and denitrifying yield (YN_2) of 0.61 after 95 h of incubation. These results were expected, because the sludge used was stabilized in a denitrifying CSTR fed with acetate and nitrate in steady state; thus, it contained an active population of organotrophic denitrifiers. Nitrogen balance showed that nitrate was mainly recovered as N_2 (around 73 %) with accumulation of NO_2 (close to 16 %). Total nitrogen recovery was of

82 %, indicating that 18 % of consumed nitrogen followed another fate (Table 2). Biomass was analyzed at the end of the batch assays, but not growth was detected. Thus, nitrogen used for the anabolic process was negligible suggesting that the metabolism was mainly catabolic. Nitrous oxide was detected after 75 h of culture (not quantified), and nitrogen compounds consumption was stopped around 90 h. The shut off of the respiratory process might be associated to nitrous oxide formation. Beristain Cardoso et al. [25] showed an inhibition of the denitrifying process due to the presence of N_2O during phenol oxidation. On the other hand, Firth and Edwards [26] have suggested that elevated concentrations of N_2O might exert a negative feedback effect upon the enzymes nitrite or nitrate reductases, switching off the metabolic pathway.

p-Cresol and Ammonium Oxidation

The *p* cresol was completely consumed by anaerobic sludge under these environmental conditions (Fig. 1b). Further analyses revealed that it was converted to *p*OHBzate, *p*OHBzalc, an unidentified intermediate and HCO_3 during the first 10 h of incubation. The total carbon recovered at the end of the batch cultures was 73 %. Thus, 27 % of consumed carbon ended up in the form of unidentified compounds (Table 2). Methane was not detected in the gas phase, thus confirming a denitrifying profile. The balance gap might be due to the accumulation of fermented products derived from *p* Cr degradation. After the first 30 h of incubation, an electron balance was made, showing that nitrite and N_2 production required approximately 12.5 mEq (milliequivalents of electrons)/L. Moreover, the electrons generated from *p* cresol oxidation to HCO_3 and *p*OHBzate were around 5 mEq/L. In order to produce the amount of nitrite and N_2, a fraction of electrons should have come from *p* Cr oxidation to the carbona ceous intermediates (~7.0 mEq/L). The electron balance suggested that the intermediate production provided the greatest number of electrons to reduce nitrate. *p* Cr consumption was linked to nitrate reduction, but nitrite accumulation occurred, suggesting that nitrite reduction was the main rate limiting step on this respiratory process. Nitrite accumulation under denitrifying conditions has been observed by several researchers using phenol or other aromatic compounds [27 29]. For instance, Thomas et al. [29] suggested that nitrite accumu lation is owing to the presence of nitrate, which exerted repression on the nitrite reductase. On the other hand, Meza Escalante et al. [23] also observed nitrite accumulation in batch tests amended with nitrate and *p* cresol, using whole cells. These authors displayed a global balance of total carbon; therefore, it is unclear whether the nitrite accumulation was due to presence of *p* Cr, or by the accumulation of phenolic intermediates. In the present work, *p* Cr was partially

oxidized to an unidentified carbonaceous intermediate, which was detected by HPLC. The unidentified intermediate reached a maximum peak around 40 h (Figs. 1b and 2). A spectrophotometric screening from 190 to 350 nm of this unknown compound was made, showing that its chemical structure was formed by a benzene ring (206.4 nm) and two substituent groups (282.4 and 299.2 nm) (Supplementary Fig. 2). Bossert and Young [30] elucidated the *p* Cr biochemical pathway using the denitrifying bacterial isolate PC 07 under nitrate reduction conditions, with *p* Cr, *p* OHBalc, and *p* OHBzald as electron donors. This model pathway involves the initial formation of a quinone, then oxygen from water is incorporated by the enzyme metilhydrolase to form *p*OHBzalc, followed by dehydrogenation to produce *p*OHBzald, and finally a new dehydrogenation is carried out to produce *p*OHBzate. Nitrate acts as external electron acceptor, and it is finally reduced to N_2. In the present work, the compound partially identified might be positioned between *p* Cr and *p*OHBzalc, and it seems to be a phenolic compound.

In the time interval between 40 and 60 h, N_2 production stopped, which might be due to the presence of this carbonaceous intermediate. For example, Thomas et al. [29] showed that there is a hindrance to degrade compounds when two substituent groups on the aromatic ring are in the ortho position to one another, such as catechol, 2,5 dihydroxybenzoic acid, *o* hydroxybenzoic acid, among others. Nitrate and ammonium were also not consumed during the same period of time; nonetheless, this compound partially identified was progressively decreasing, suggesting some biotransformation for another via not associated to denitrification.

Regarding to the second reducing source, ammonium, there was a lag phase of 30 h before being consumed (Fig. 1a). Ammonium consumption efficiency was of 83 %, and it was oxidized at specific rate of 1.02±0.07 mg NH_4^+ N/g VSS h. The kinetic results showed that denitrifying sludge consumed ammonium faster than nitrate (Table 3). On the other hand, nitrate reduction was not linked to ammonium oxidation, suggesting that sludge was not able to use nitrate as terminal electron acceptor for the anaerobic ammonium oxidation. In fact, Van de Graaf et al. [14] carried out batch experiments with tracers, showing that the actual electron acceptor for anaerobic ammonium oxidation was nitrite instead of nitrate, being N_2 and NO_3 the end products. According to the experimental data, anaerobic sludge oxidized ammonium when nitrite was transiently accumulated, in agreement to the anammox biological process. Further, Sun et al. [31] observed anammox activity in several sludge samples taken from municipal wastewater treatment plants (WWTPs), but the response time to produce N_2 took more than 1 month. In the present work, N_2 production from the anaerobic ammonium oxidation was relatively fast when nitrite was produced. Similar results were previously reported by Gonzalez Blanco et al. [20], who evaluated the kinetics of ammonium oxidation from denitrifying sludge metabolically stable using nitrite as terminal electron acceptor; observing a faster N_2 production without lag phase. Therefore, in order to remove different pollutants by using denitrifying sludge metabolically stable might be a good strategy to startup bioreactors for simultaneous removal of nitrogenous pollutants, such as ammonium and nitrite, as well as organic compounds, such as phenolic compounds.

It has been well established that the anammox metabolism requires nitrite as the terminal electron acceptor. As nitrite was not externally fed to the batch assays, it must have been internally produced by the biochemical reactions involved, for example during the nitrate partial reduction linked to *p* Cr oxidation (Fig. 3). In parallel, a simultaneous consumption of
p Cr/NO_3 or NH_4^+/NO_2 was observed, suggesting strongly the synergy of anaerobic ammonium oxidation and organotrophic denitrification. In the last decade, there are several reports about the coupling between anammox and denitrification, where nitrite has been promoted in bioreactors of partial nitrification to be used after in anammox bioreactors: observing a competition for the nitrite reduction between anammox bacteria and denitrifiers [19]. For example, Shi et al. [32] observed in a membrane biofilm reactor fed with methane, ammonium, and nitrate, an occurrence of anammox coupled to denitrification. Denitrifying bacteria reduced nitrate to nitrite, using electrons derived from methane, afterward nitrite was used by anammox bacteria. Finally, in the present work, the coupling between two respiratory processes; anaerobic ammonium oxidation and organotrophic denitrification might be a good strategy for treating industrial wastewaters containing nitrate, ammonium, and phenolic compounds, like those generated from petrochemical industry or found in groundwaters.

Conclusions

Nitrate, ammonium, and *p* cresol were removed at 92, 83, and 100 %, respectively, being the main products N_2 and CO_2. Incomplete *p* cresol oxidation led to the formation of an uniden tified intermediate, which induced nitrite accumulation promoting the anaerobic ammonium oxidation process. Thus, two respiratory processes; organotrophic denitrification and anaerobic ammonium oxidation took place in a sequential way after 90 h. Finally, the presence of the unknown phenolic compound inhibited the respiratory process of denitrification, but induced the ammonium consumption owing to nitrite accumulation.

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