Biological removal of ammonium and *p*-cresol linked to nitrite reduction

G. González-Blanco*, R. Beristain-Cardoso*, F. Cuervo-López*, F.J. Cervantes** and J. Gomez*,

* Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, DF, Mexico
(E-mail: *gehovan25@yahoo.com.mx*; *becr@xanum.uam.mx; fmcl@xanum.uam.mx; dani@xanum.uam.mx*)
** División de Ciencias Ambientales, Instituto Potosino de Investigación Científica y Tecnológica (IPICyT), San Luis Potosí, Mexico
(E-mail: *fjcervantes@ipicyt.edu.mx*)

Abstract
The metabolic capability of denitrifying sludge to oxidize ammonium and *p*-cresol was evaluated in batch cultures. Ammonium oxidation was studied in presence of nitrite and/or *p*-cresol by 55 hours. At 50 mg/L NH_4^+-N and 76 mg/L NO_2^--N, the substrates were consumed at 100% and 95%, respectively, being N_2 the product. At 50 mg/L NH_4^+-N and 133 mg/L NO_2^--N, the consumption efficiencies decreased to 96% and 70%, respectively. The increase in nitrite concentration affected the ammonium oxidation rate. Nonetheless, the N_2 production rate did not change. In organotrophic denitrification, the *p*-cresol oxidation rate was slower than ammonium oxidation. In litho-organotrophic cultures, the *p*-cresol and ammonium were oxidized simultaneously but at different specific rates, being the main products, N_2 and HCO_3^-. Finally, this is the first work reporting the simultaneous oxidation of ammonium and *p*-cresol linked to nitrite reduction.

Keywords: Ammonium, *p*-cresol, oxidation, litho-organotrophic

INTRODUCTION

Industrial wastewaters from chemical and petrochemical plants entering a water body represent a great challenge for treatment. The major part of wastewaters polluted with *p*-Cresol derives from petrochemical industry where nitrite and ammonium are also found (Olmos et al., 2004). *p*-Cresol is extremely toxic, corrosive and causes nervous system depression (Schepers et al., 2007). Nitrite negatively affects human health and cause severe problems to aquatic ecosystems, whereas ammonium can causes eutrophication (Camargo and Alonso, 2006).

Nitrite is an intermediary in the denitrification, while it is a substrate for the anammox (anaerobic ammonium oxidation) process. Anammox bacteria oxidize ammonium to N_2 using nitrite as electron acceptor. Nonetheless, high concentration of organic matter can affect the anammox process (Dapena-Mora et al., 2007). On the other hand, denitrification is a biological process involving fourth enzymatic steps in which nitrate is reduced to nitrite, nitric oxide, nitrous oxide and finally to N_2. In literature has been reported the potential of denitrification for oxidizing phenolic compounds (Meza-Escalante et al., 2008; Beristain-Cardoso et al., 2009) and there are authors suggesting that the coexistence of denitrification and anammox might allow the simultaneous removal of ammonium in presence of simple organic matter (Kartal et al., 2007).

Meza-Escalante et al. (2008) reported that nitrite can be accumulated from nitrate partial reduction due to the presence of phenolic compounds. The transitory nitrite formation from organotrophic denitrification might be a good strategy for ammonium oxidation. The oxidation of ammonium and the mineralization of phenolic compounds require different types of reactors connected in series. The use of one only bioreactor for wastewater treatment will be a feasible process. The goal of this research was to evaluate the metabolic capability of denitrifying sludge for ammonium and *p*-cresol oxidation linked to nitrite reduction. This research might be crucial because the real wastewaters are highly heterogeneous.

MATERIALS AND METHODS

Batch cultures: The denitrifying sludge used for the batch cultures was cultivated in a bioreactor (5 L) utilizing a feed containing acetate (2g/L) and nitrate (1.84g/L). Batch cultures were conducted in 160 mL serological bottles containing 60 mL of liquid medium. The medium was composed as follows (g/L): K_2HPO_4 (4.5), KH_2PO_4 (3.0), and trace elements solution supplied at 1.5 ml/L. Trace element solution contains (g/L): EDTA (5), $CuSO_4 \cdot 5H_2O$ (1.57), $CaCl_2 \cdot 2H_2O$ (5.54), $MnCl_2$ (5.0), $(NH4)_6Mo_7O_{24} \cdot 4H_2O$ (1.1), $FeCl_3$ (05.0), $CoCl_2 \cdot 6H_2O$ (1.6), $MgCl_2$ (5.0). Bioassays were conducted by duplicate, and incubated for 55 h at 30°C in a shaker at 200 rpm. Batch cultures were inoculated with 2.0 ± 0.2 g VSS/L. The initial and final pH for all batch cultures was of 7.0 ± 0.5. The average specific rates (q) were calculated for the integrated Gompertz model (Beristain-Cardoso et al., 2011). The coefficient of determination was higher than 0.95 for all cases. Microbial activity was evaluated in terms of consumption efficiency (E, [g of N or C consumed/g of N or C fed] X 100), production yield (Y, [g of N or C produced/g of N or C consumed]) and a specific consumption rate (q, [mg of substrate or product/g VSS d]).

Analytical methods: NH_4^+ was analyzed by a selective electrode. NO_2^- and NO_3^- were analyzed by capillary electrophoresis. The *p*-cresol and its intermediaries were analyzed by using liquid chromatography. The soluble inorganic carbon was measured using a TOC analyzer. N_2, N_2O and CO_2 were analyzed by using a gas chromatograph. The volatile suspended solids (VSS) were conducted according to Standard Methods (APHA, 2005). Standard curves were drawn in triplicates for each analytical method. In all cases, the coefficient of variation was less than 10%.

RESULTS AND DISCUSSION

***Lithotrophic denitrification*:** Batch cultures were spiked with 50 mg/L NH_4^+-N and 76 mg/L NO_2^--N or 133 mg/L NO_2^--N. At 76 mg/L NO_2^--N (Fig. 1A), ammonium (E_{NH4+}) and nitrite (E_{NO2-}) consumption efficiencies were of 100%, achieving N_2 yield (Y_{N2}) of 0.88 mg/mg NH_4^+-N consumed. NH_4^+-N (q_{NH4+}) and NO_2^--N (q_{NO2-}) specific consumption rates were of 32.5 and 44 mg N/g VSS h, respectively. At 133 mg/L NO_2^--N, E_{NH4+} was near to 100%, but E_{NO2-} and Y_{N2} diminished at 26% and 12.5%, respectively. The q_{NH4+} and q_{NO2-} were of 21.6 and 32.5, respectively. The diminishing on ammonium consumption rate suggested that nitrite might be acting as inhibitory agent. For instance, Strous et al. (1999) and Dapena-Mora et al. (2007) observed an inhibitory effect on ammonium oxidizing activity at concentrations higher than 100 mg NO_2^--N/L. Rake and Eagon (1980) suggested that nitrite is an uncoupler of the respiratory chain. Thus, in spite of being nitrite a substrate for ammonium oxidation it can also have a negative effect at certain culture conditions. On the other hand, an increase in initial nitrite concentration did not affect the specific N_2 production rates (~ 61 mg N_2/g VSS d for both studies). The specific N_2 production rates obtained from a denitrifying sludge physiologically stable were six-fold lower than reported by Jetten et al. (1998) and Egli et al. (2001) who reported values of specific N_2 production rates between 0.02 and 0.046 mg/mg protein h (~ 300-690 mg N_2/g VSS d) by using anammox sludge. It is important to note that several factors might be affecting the production rate values, such as history of sludge, kind of sludge, pH and substrate concentration, among others. Nonetheless, it is worth to remark that the denitrifying sludge physiologically stable used in this work showed the metabolic capability for molecular nitrogen production from ammonium and nitrite.

Organotrophic denitrification: Batch cultures were spiked with 49 mg/L *p*-cresol-C (pCr) and 94.6 mg/L NO_2^--N. E_{NO2-} and E_{pCr} were around 40%. Nitrite was completely reduced to N_2, and the phenolic compound was mineralized, achieving a bicarbonate yield (Y_{HCO3-}) of 1.0 mg HCO_3^--C/ mg pCr consumed. Specific *p*-cresol consumption rate (q_{pCr}) was of 3.6 mg C/g VSS h, while the q_{NO2-} and q_{N2} were of 8.88 and 3.84, respectively. The denitrifying sludge showed the metabolic capability for oxidizing *p*-cresol using nitrite as electron acceptor. In comparing the lithotrophic and organotrophic denitrifying rates, the denitrifying sludge oxidizes the ammonium ten-fold faster than

p-cresol. For instance, Meza-Escalante et al. (2008) studied the *p*-cresol oxidation under denitrifying conditions in batch cultures, but using nitrate as electron acceptor. These authors reported that phenolic compound was oxidized linked to nitrate reduction, with specific N_2 production rate of 5.9 ± 1.6 mg/gVSS d. In contrasting this last value with the value obtained in the present study, it is indicating that the organotrophic denitrification is a slow biochemical reaction. The low consumption rate of *p*-cresol compared to the ammonium oxidation rate might be related to the affinity constant (Ks) value of *p*-cresol, possibly higher than for ammonium. Nonetheless, the denitrifying consortium showed metabolic capability for oxidizing *p*-cresol linked to nitrite reduction to N_2. It is important to note that the information on this topic is still scarce. Thus, it is necessary to carry out more detailed studies in order to understand better the physiological and kinetics behavior.

Litho-organotrophic denitrification*:* Batch cultures were spiked with 50 mg/L pCr, 53 mg/L NH_4^+-N and 76 mg/L NO_2^--N. E_{NO2-} and E_{NH4+} diminished 2.34 and 1.65 times, respectively, in regarding to lithotrophic culture. The main products were HCO_3^- and N_2 (Fig. 1B), with high product yields. The kinetics of nitrite and ammonium consumption and N_2 production also diminished 4.31, 3.13 and 4.2 times, respectively, in regarding to the lithotrophic cultures. These experimental results clearly indicated that *p*-cresol exerted a negative effect on the physiology and kinetics of litho-organotrophic denitrification. On the other hand, nitrate formation was detected under this culture conditions. In this sense, Jetten et al. (1998) pointed out that nitrate coming from nitrite oxidation is only produced with the aim to generate reducing equivalents necessary for the reduction of CO_2. In the present work, biomass growth was not detected by the analytical method used. Likewise, the HCO_3^- was not consumed when nitrate was formed. These results might indicate that nitrate formation was no linked to the anabolic pathway. Regarding to *p*-cresol, it was consumed at 18.9%, being the end product HCO_3^-. The specific *p*-cresol oxidation rate diminished around 25%, in comparison to the organotrophic denitrifying culture. The denitrifying sludge oxidized the ammonium 3.7-fold faster than *p*-cresol. This might suggest a competition between both substrates, thus affecting the specific consumption rates either *p*-cresol or ammonium. Anyway, these evidences showed that ammonium and *p*-cresol were simultaneously oxidized in presence of nitrite, but at different specific rates.

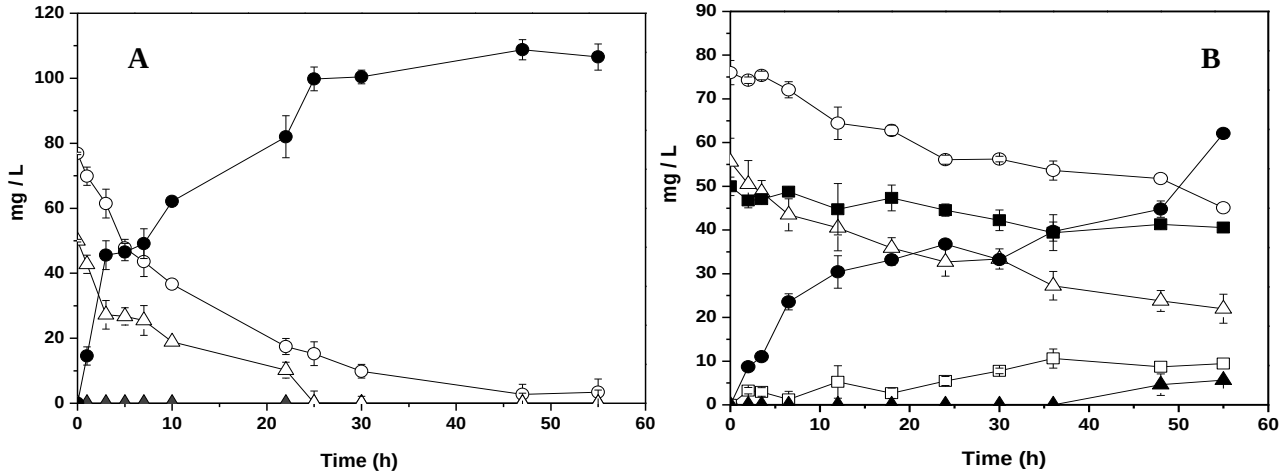

Figure 1 (A) Time course of ammonium oxidation and N_2 production by denitrifying sludge, in batch lithotrophic cultures. (B)Time course of *p*-cresol and ammonium oxidation by denitrifying sludge, in batch litho-organotrophic cultures. (○) NO_2^--N, (Δ) NH_4^+-N, (▲) NO_3^--N, (●) N_2, (■) *p*-cresol-C, (□) HCO_3^--C.

CONCLUSIONS

In lithotrophic cultures with ammonium and nitrite, the denitrifying sludge removed efficiently the ammonium and nitrite, resulting as end product, molecular nitrogen. In litho-organotrophic cultures,

the denitrifying sludge showed the metabolic capability for oxidizing simultaneously ammonium and *p*-cresol, but at different specific rates. Finally, this is the first work reporting the simultaneous oxidation of ammonium and *p*-cresol with the concomitant production of N_2 from denitrifying sludge physiologically stable.

ACKNOWLEDGEMENTS

This work was supported by CONACyT (CB-2011-01, 164746 SEP-CONACyT). González-Blanco G., received a Ph.D fellowship from CONACyT-México.

REFERENCES

APHA/AWWA/WEF, 2005. Standard Methods for the Examination of Water and Wastewater, Washington DC, USA.

Beristain-Cardoso, R., Texier, A.-C., Sierra-Alvarez, R., Razo-Flores, E., Field, J.A., Gómez, J., 2009. Effect of initial sulfide concentration on sulfide and phenol oxidation under denitrifying conditions. Chemosphere. 74, 200-205.

Beristain-Cardoso, R., Gómez, J., Mendéz-Pampín, R., 2011. Sulfide and ammonium oxidation, acetate mineralization by denitrification in a multipurpose UASB reactor. Bioresour. Technol. 102, 2549-2554.

Camargo, A., Alonso, A., 2006. Ecological and toxicological effects of inorganic nitrogen pollution in aquatic ecosystems: a global assessment. Environ. International. 32, 831-849.

Dapena-Mora, A., Fernández, I., Campos, J.L., Mosquera-Corral, A., Méndez, R. Jetten, M.S.M., 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production, Enzyme Microb. Technol. 40, 859-865.

Egli, K., Franger, U., Alvarez, P.J.J., Siegrist, H., Vandermeer, J.R., Zehnder, A.J.B., 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch. Microbiol. 175, 198-207.

Jetten, S.M., Strous, M., van de Pas-Schoonen, K.T., Schalk, J., van Dongen, G.J.M., van de Graaf, A.A., Logemann, S., Muyzer, G., van Loosdrecht, M.C.M., Kuenen, J.G., 1998. The anaerobic oxidation of ammonium. FEMS Microbiol. Rev. 22, 421-437.

Kartal, B., Rattray, J., van Niftrik, L., van de Vossenberg, J., Schmid, M., Webb, R.I., et al. 2007. Candidatus 'Anammoxoglobus propionicus' gen. nov., sp. nov., a new propionate oxidizing species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. 30, 39-49.

Meza-Escalante, E.R., Anne-Claire, T., Cuervo-López, F., Gómez, J., Cervantes, F., 2008 Inhibition of sulfide on the simultaneous removal of nitrate and *p*-cresol by a denitrifying sludge. J. Chem. Technol. Biot. 83, 372-377.

Olmos, A., Olguin, P., Fajardo, C., Razo-Flores, E., Monroy, O., 2004 Physicochemical characterization of spent caustic from the OXIMER process and source waters from Mexican oil refineries. Energy Fuels. 18, 302-304.

Rake, J.B., Eagon, R.G., 1980. Inhibition, but not uncoupling, of respiratory energy coupling of three bacterial species by nitrite. J. of Bateriol. 144, 975-982.

Schepers, E., Meert, N., Glorieux, G., Goeman, J., Van der Eycken, J., Vanholder, R., 2007. *p*-cresylsulphate, the main in vivo metabolite of *p*-cresol, activates leucocyte free radical production. Nephrol. Dial Transplant. 22, 592-596.

Strous, M., Kuenen, J.G., Jetten, M.S.M., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248-3250.