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ANAEROBIC AMMONIUM OXIDATION IN A BIOREACTOR TREATING SLAUGHTERHOUSE WASTEWATER

V. Reginatto[1*], R. M. Teixeira[1], F. Pereira[1], W. Schmidell[1], A. Furigo Jr[1], R. Menes[2], C. Etchebehere[2] and H. M. Soares[1]

[1]Chemical and Food Engineering Department, Technological Center, Federal University of Santa Catarina, Campus Universitário, Trindade, Phone: +55-48-331-9448, Fax: +55-48-331-9687, P.O. Box 476, CEP 88040-900, Florianópolis - SC, Brazil.
E-mail: reginatto@enq.ufsc.br
[2]Cátedra de Microbiologia , Facultad de Química y Facultad de Ciencias, Universidad de la República. CC 1157, Montevideo, Uruguay.
E-mail: jmenes@fq.edu.uy, E-mail: cetchebe@fq.edu.uy

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Abstract - Ammonium oxidation was thought to be an exclusively aerobic process; however, as recently described in the literature, it is also possible under anaerobic conditions and this process was named ANAMMOX. This work describes the operation of a system consisting of a denitrifying reactor coupled to a nitrifying reactor used for removal of nitrogen from slaughterhouse wastewater. During operation of the denitrifying reactor an average nitrogen ammonium removal rate of 50 mg/Ld was observed. This biomass was used to seed a second reactor, operated in repeated fed batch mode, fed with synthetic medium specific to the growth of bacteria responsible for the ANAMMOX process. The nitrogen loading rate varied between 33 and 67 mgN/Ld and average nitrogen removal was 95% and 40%, respectively. Results of fluorescence *in situ* hybridization (FISH) confirmed the presence of anammox-like microorganisms in the enriched biomass.
Keywords: Nitrogen removal; Anaerobic ammonium oxidation; ANAMMOX; Slaughterhouse wastewater.

INTRODUCTION

The nitrogen in industrial wastewater is mainly found in organic forms like protein or urea, which form ammonium following hydrolysis (Mudrack and Kunst, 1994). Ammonium nitrogen discharged into the environment is extremely harmful due to the high toxicity of free ammonia at a pH higher than 8.0 and also because of its oxygen demand in bodies of water (EPA, 1975).

Until few years ago the only known way to remove nitrogen from wastewater by biological processes was with a system of nitrification and denitrification. In nitrification, first ammonium is oxidized to nitrite by the nitritation process and then to nitrate, mainly by autotrophic bacteria of the genera *Nitrosomonas* and *Nitrobacter*. In denitrification, heterotrophic bacteria reduce nitrate in the absence of oxygen, using organic matter as electron donor, producing intermediates like NO_2, NO, N_2O and finally N_2 gas, which is removed from the liquid phase (Etchebehere et al., 2001).

In 1995 in Holland, Mulder and co-workers observed for the first time ammonium oxidation under anaerobic conditions and named this process Anaerobic Ammonium Oxidation or ANAMMOX. Years prior to the discovery of this process, based on thermodynamic studies, Broda (1977) foresaw the possibility of microorganisms able to oxidize ammonium using nitrate or nitrite as electron acceptor. According to the ANAMMOX stoichometry proposed by van Dongen et al. (2001),

*To whom correspondence should be addressed

shown in Equation 1, the theoretical rate of substrate consumption (NO_2^-/NH_4^+) is 1.32, while the rate of nitrate formation and ammonium consumption (NO_3^-/NH_4^+) is 0.26.

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + \\ +0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + \\ +0.066\,CH_2O_{0.5}N_{0.15} + 2.03\,H_2O \quad (1)$$

Microorganisms responsible for anaerobic ammonium oxidation were identified as belonging to the phylum *Planctomycetes* and are as Candidatus *Brocadia anammoxidans* (Strous et al., 1999), Candidatus *Kuenenia stutgartiensis*, Candidatus *Scalindua wagneri* and Candidatus *Scalindua brodae* (Kuypers et al., 2003). The first two have been found in wastewater treatment systems. *Scalindua* has also been detected in many marine ecosystems.

In the last several years, there have been an increasing number of articles in the specialized literature showing the advantages of the ANAMMOX process over the traditional one, such as less need for aeration for nitrification and occasionally the addition of organic matter for denitrification. In addition, costs related to sludge disposal are reduced because of the lower rate of growth of the microorganisms responsible for ANAMMOX than of the ones responsible for the nitrification/denitrification process (Ye and Thomas, 2001).

In Brazil this new process in anaerobic reactors has not yet been described. Thus, this work describes the capacity of a biomass from a nitrification-denitrification reactor system used to treat slaughterhouse wastewater to remove nitrogen by the ANAMMOX process.

MATERIAL AND METHODS

Nitrification-Denitrification System Set-up

The system was composed of two reactors with continuous flow operated in series: a hybrid anaerobic reactor connected to an upflow biological filter with submerged aeration with recycling for the hybrid reactor. The two reactors were constructed of glass with a diameter of 6cm and a height of 40 cm and were filled with corrugated pipes of PVC with a ½ inch diameter. The aerobic and anaerobic reactors have 790 mL and 745 mL of effective volume, respectively. The reactors had been connected according to Figure 1.

System Operation

Inoculum used for the hybrid reactor was from an anaerobic stabilization pond at a chicken and swine processing plant and that for the aerobic reactor was from a domestic wastewater treatment plant. Nitrifying and denitrifying biomasses were enriched by feeding with synthetic medium during reactor start-up, as described elsewhere (Teixeira et al., 2000). After the start-up period, the reactor system was fed with wastewater from a slaughterhouse pretreated in an anaerobic stabilization pond, whose composition is shown in Table 1. A peristaltic pump containing two headstocks provided an outflow of 475 mL/d with a recycle ratio (R) of 1.8.

Table 1: Chemical characterization of slaughterhouse wastewater

Component	
$N\text{-}NH_4^+$ (mg/L)	163.7 (±31.6)
$N\text{-}NO_3^-$ (mg/L)	nd
$N\text{-}NO_2^-$ (mg/L)	nd
COD (mgO_2/L)	614.5 (± 130.4)
pH	7.20 (± 0.32)

nd: not detected

Mass Balance for the Denitrifying Reactor

The efficiency of the denitrifying reactor in removing chemical oxygen demand (COD) and nitrogen as ammonium ($N\text{-}NH_4^+$) and nitrate ($N\text{-}NO_3^-$) depends on the recycle ratio. Equation 2 represents COD and nitrogen mass balance for the denitrifying reactor for the steady state period, for the reactor configuration shown in Figure 1.

$$r\,V = Q[C]_e + RQ[C]_m - (Q + RQ)[C]_d \quad (2)$$

r: removal rate of $N\text{-}NH_4^+$, $N\text{-}NO_x^-$ or COD in the denitrification reactor (mgL/d);
V: reactor volume (L);
Q: flow rate (L/d);
$[C]_e$: $N\text{-}NH_4^+$, $N\text{-}NO_x^-$ or COD concentration in influent (mg/L);
$[C]_m$: $N\text{-}NH_4^+$, $N\text{-}NO_x^-$ or COD concentration in the

recycle (mg/L);
$[C]_d$: $N\text{-}NH_4^+$, $N\text{-}NO_x^-$ or COD concentration in the effluent (mg/L);
R: recycle ratio (=1.8)

Enrichment of Denitrifying Sludge in Microorganisms Responsible for Anaerobic Ammonium Oxidation

After 200 days of operation of the nitrification-denitrification reactor system, sludge from the denitrifying reactor was taken and used to seed an anaerobic Repeated Fed Batch (also called a sequencing batch reactor – SBR) reactor with a 250 mL volume, which was operated for 160 days with total cell retention. The denitrifying sludge was fed with an autotrophic synthetic medium containing ammonium, nitrite, bicarbonate and micronutrients proposed by van de Graaf et al. (1996). The reactor was fed daily using a syringe to remove a specific volume from the reactor and feed in the same volume of fresh synthetic medium. The volume fed in varied from 40 to 80ml per day during the period of operation, varying nitrogen loading from 33 to 67 mgN/Ld. The anaerobic conditions were assured by flushing with helium for 20 minutes and sealing the reactor with butyl rubber stoppers. The pH was kept between 7.5 and 8.0 using NaOH or HCl 01N, and the temperature was maintained at 35 $^oC \pm 0.5^oC$.

Analytical Methods

Ammonium nitrogen ($N\text{-}NH_4^+$) was analyzed by the colorimetric method of Nessler according to Vogel (1981). Nitrite was determined colorimetrically with the Nitriver kit (HACH® Company) and nitrate was determined by the method of acetyl salicylic acid (Cataldo et al., 1975). The chemical oxygen demand (COD) was determined by the method of closed reflux according to *Standard Methods for Examination of Water and Wastewater* (APHA, AWWA, WEF, 1995). The N_2 was measured by gas chromatography (Perkin-Elmer 1B) with a thermal conductivity detector and a Porapak Q® column.

Fluorescence in Situ Hybridization (FISH) and Microscopy

Samples taken at the end of the period of operation were fixed by adding three volumes of 4% (w/v) paraformaldehyde in PBS (130 mM NaCl, 10 mM sodium phosphate) for 3 h at 4°C. Then the cells were washed twice with PBS, resuspended in PBS:ethanol (1:1) and stored at –20°C. For enumeration, the biomass was resuspended in sodium pyrophospate ($2.8gL^{-1}$) and homogenized for 1 min with an ultrasonic probe. Five μl of fixed samples were spotted onto microscopic slides and sequentially dehydrated and hybridized with a Cy3-labeled probe (Amx820) in accordance with Egli et al. (2003). Slides were incubated in the washing buffer with 0.1 mgL^{-1} of 4,6-diamidino-2-phenylindole (DAPI) for 10 min at 48°C, dried and observed with an Olympus BX41 microscope equipped with MWU (DAPI) and MWIG (Cy3) filters. Digital images were captured with a CCD camera and acquired with the program Image-ProPlus (MediaCybernetics Inc., MD, USA).

RESULTS AND DISCUSSIONS

Denitrifying Reactor

This work addresses nitrogen loss in the denitrifying reactor (anaerobic), and the mass balances of the wastewater components are shown only for the denitrifying reactor, keeping in mind that it was coupled to the aerobic one, as shown in Figure 1.

In Figures 2 and 3 the inflow and outflow of COD and nitrate concentrations and their removal, are represented. The denitrifying reactor should be able to reduce nitrate coming from the aerobic reactor to nitrogen gas using wastewater COD as electron donor; however this was not the only nitrogen elimination metabolic pathway. In Figure 2 it can be observed that COD removal in the denitrifying reactor oscillated with COD concentration in the influent. After 100 days COD removal remained about 170mgCOD/Ld. Denitrification stoichometry proposes that about 3.7mg COD are necessary to reduce 1.0mg $N\text{-}NO_3^-$ (Henze et al., 1997); thus the amount of COD consumed should be able to denitrify around 46mgN/Ld of $N\text{-}NO_3^-$. However, as can be verified in Figure 3, during the same period (between 100 and 200 days) the mean nitrate removal in the denitrifying reactor was around 17mgN/Ld, much lower than the expected value, particularly from the 140th day, when the removal was only about 11mg$N\text{-}NO_3^-$/Ld.

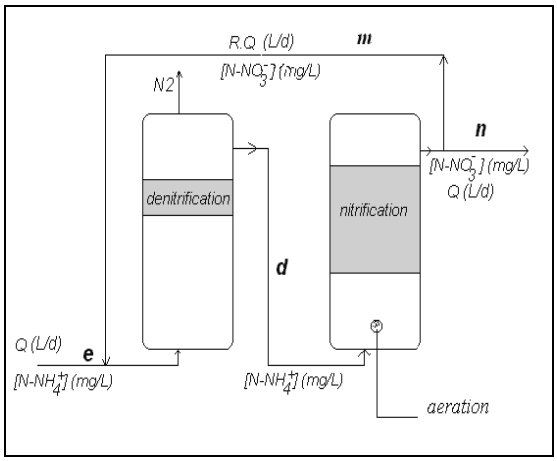

Figure 1: Scheme of experimental reactors

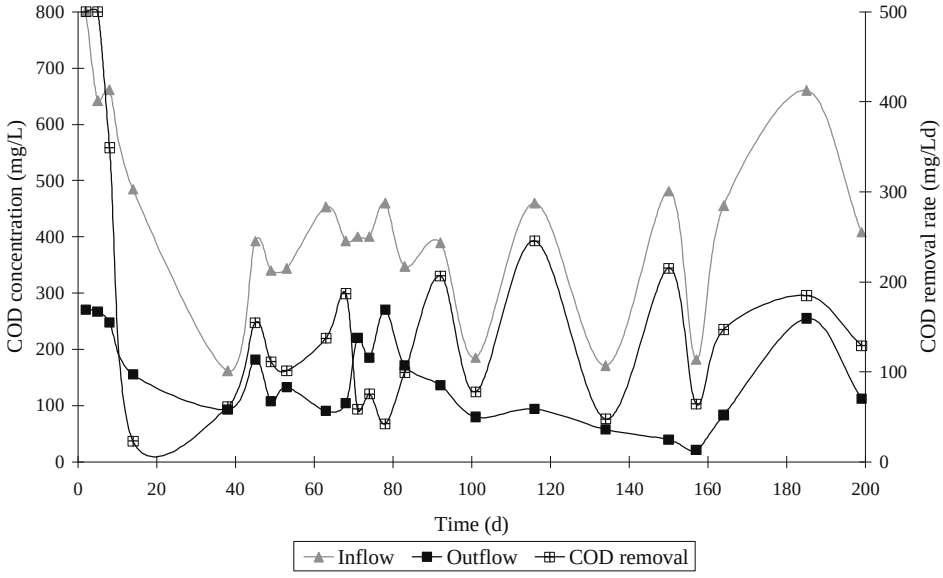

Figure 2: COD concentration in the influent and effluent and removal rate in the denitrifying reactor.

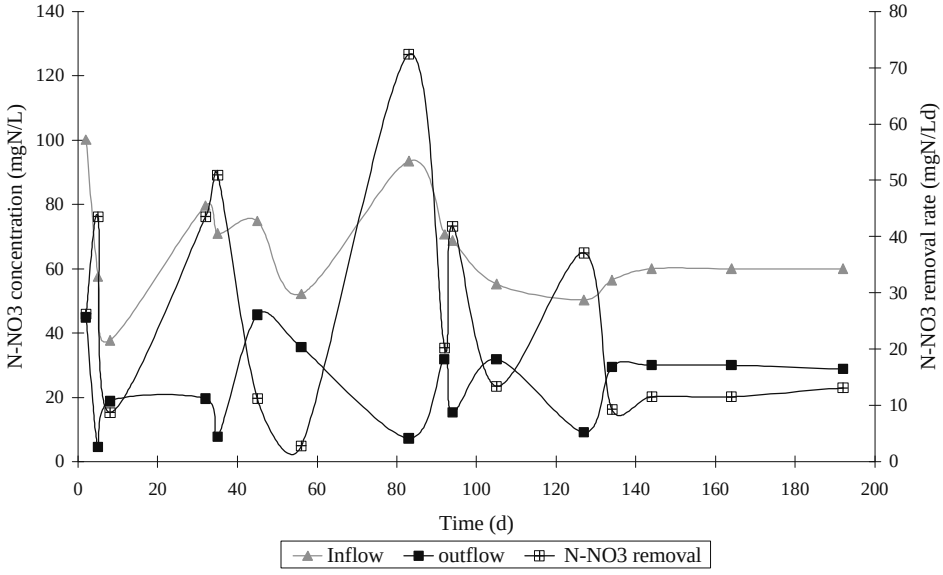

Figure 3: $N\text{-}NO_3^-$ concentration in the influent and effluent and removal rate in the denitrifying reactor.

Results described above suggest the difficulty to establish conventional heterotrophic denitrification, which could be attributed to different factors, as discussed below.

The COD consumption observed in the denitrifying reactor (Fig. 2) could take place by anaerobic digestion, which could possibly occur together with anaerobic heterotrophic denitrification.

Inhibition of one of the denitrification steps, generating an intermediate should also be taken into account. The possible inhibition of denitrification by a wastewater compound was not examined in depth in this work, but if this process were inhibited, intermediates like $N\text{-}NO_2^-$ could be produced in the reactor and quickly reduced by an alternative electron donor, for example ammonium ion in the case of the ANAMMOX process.

Another possibility is that the influent could bring into the denitrifying reactor dissolved oxygen from nitrification or from the feeding that would be used to cause nitritation, i.e., to oxidize part of the $N\text{-}NH_4^+$ to $N\text{-}NO_2^-$, thereby allowing the ANAMMOX process to become established. In addition, if the ANAMMOX process occurs, for one of the reasons discussed above, according to the ANAMMOX stoichometry described in Equation 1, nitrate will be produced, contributing to a lower rate of nitrate removal.

In Figure 4 the concentration of ammonium nitrogen in the reactor influent and effluent and the ammonium nitrogen conversion (removal) in the denitrifying reactor are shown, taking into account the recycle coming from the aerobic reactor, in accordance with the mass balance given in Equation 2.

In Figure 4 it is shown that the ammonium nitrogen concentration in the denitrifying reactor was lower than that in the influent (see Table 1), probably due to the dilution effect of the recycle rate in the nitrification reactor. However, in accordance with the mass balance for this reactor (Equation 2), a mean ammonium nitrogen removal rate of around 50mgN/Ld was verified for the entire period monitored. This conversion was higher in the interval of 50 to 80 days reaching a maximum value of 92.4mgN/Ld. During the 150th and 200th days of operation another period of high nitrogen ammonium conversion of about 57 mgN/Ld was observed. The nitrogen incorporation of the cells to form biomass is not enough to explain this result. One of the possibilities is that the biomass may be able to cause ANAMMOX using NO_2^- that was probably produced by inhibition of denitrification or by nitritation, as discussed above. It was recently suggested by some authors that the ANAMMOX process is the cause of ammonium nitrogen losses in denitrifying reactors (Mulder et al., 1995; Jetten et al., 1999). Based on this hypothesis, part of the biomass was transferred from the denitrifying reactor to a second reactor, which was operated under optimal conditions to study the process of anaerobic ammonium oxidation.

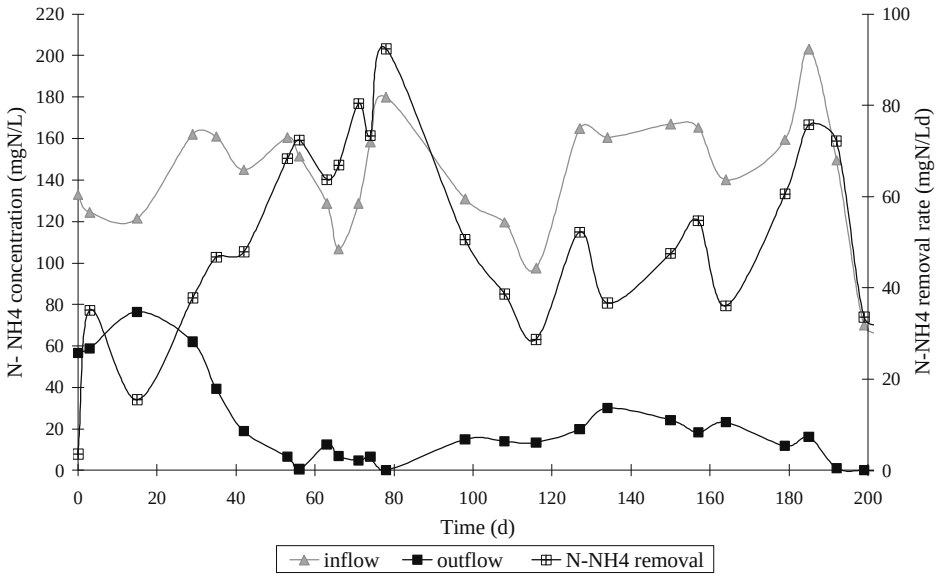

Figure 4: $N\text{-}NH_4^+$ concentration in the influent and effluent and removal rate in the denitrification reactor.

ANAMMOX Enrichment Reactor

During the enrichment of microorganisms able to cause the ANAMMOX process, an autotrophic synthetic medium as described in Materials and Methods was used. The nitrogen loading rate used in the reactor, calculated as the total amount of $N\text{-}NH_4^+$ and $N\text{-}NO_2^-$ concentrations, varied from 33 to 67 mgN/Ld, as shown in Figure 5.

During the first 19 days the nitrogen loading rate used in the reactor was 33mgN/Ld, reaching 95% nitrogen removal. Considering that the residual ammonium concentration in the reactor was low, the load was increased to 50mgN/Ld until day 40, when the load was increased to 67mgN/Ld.

In this last step, the feeding that was initially done once was changed to twice a day. This procedure aimed to reduce the overload of nitrite and ammonium during the feeding. This load was maintained for 22 days but during this time a decrease in the efficiency of nitrogen elimination was observed as can be clearly observed in Figure 5. A nitrogen loading rate of about 60mgN/Ld was maintained from day 54 to 89 in an attempt to achieve the adaptation of the microorganisms to this nitrogen load. However, the adaptation was not observed and the nitrogen loading rate had to be decreased to values lower than those used at the beginning of the operation (33mgN/Ld).

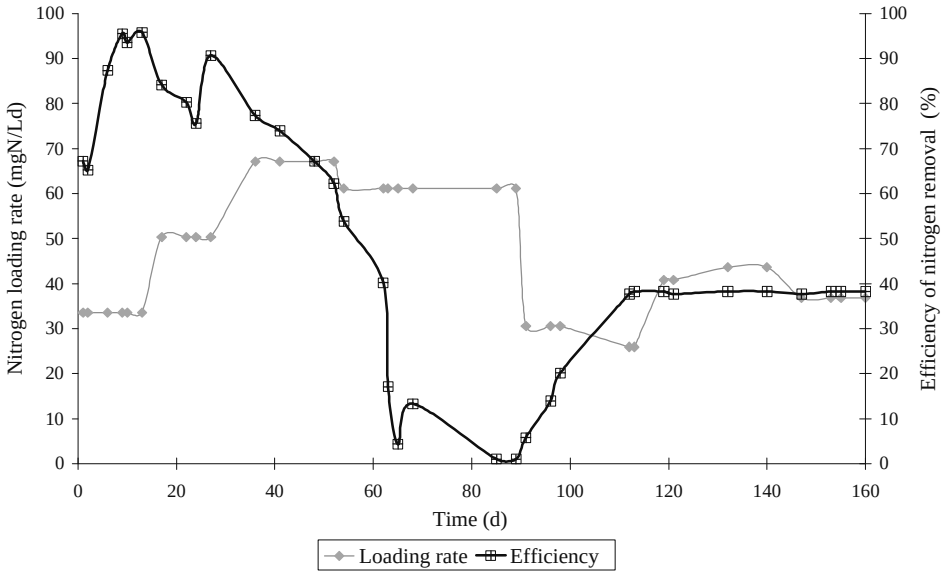

Figure 5: Nitrogen loading rate and the efficiency of nitrogen removal under anaerobic conditions.

After this alteration, the efficiency of nitrogen elimination returned to about 40 %, showing that inhibition by a higher nitrogen load was reversible. The reactor was submitted to another increase in loading rate to 40mgN/Ld and the efficiency of nitrogen elimination remained around 40 % during a period of 50 days. Gas-chromatography analysis in this phase revealed 98 % N_2 in the gas content. The concomitant consumption of nitrite and ammonium under anoxic conditions with N_2 formation suggested that the nitrogen elimination observed occurred by this new recently described metabolic pathway – ANAMMOX (Jetten et al., 1999).

To confirm this hypothesis a fluorescence *in situ* hybridization analysis was carried out with a specific probe for the group of anammox-like bacteria (Amx 820) described in the literature (Egli et al., 2003). Hybridization of the enriched biomass with the specific probe can be observed in Figure 6. The light area represents hybridization of the biomass sample with the Amx 820 fluorescently labeled probe; stained cells in dense aggregates of coccoid morphology can be seen. The cells showed an inner area with a lower signal intensity, as had been described previously for the anammox-like microorganisms (Egli et al., 2003).

Thus, these results demonstrated the nitrogen loss in a denitrifying reactor treating slaughterhouse wastewater by ANAMMOX, although it was unstable probably due to the low concentration of specific biomass.

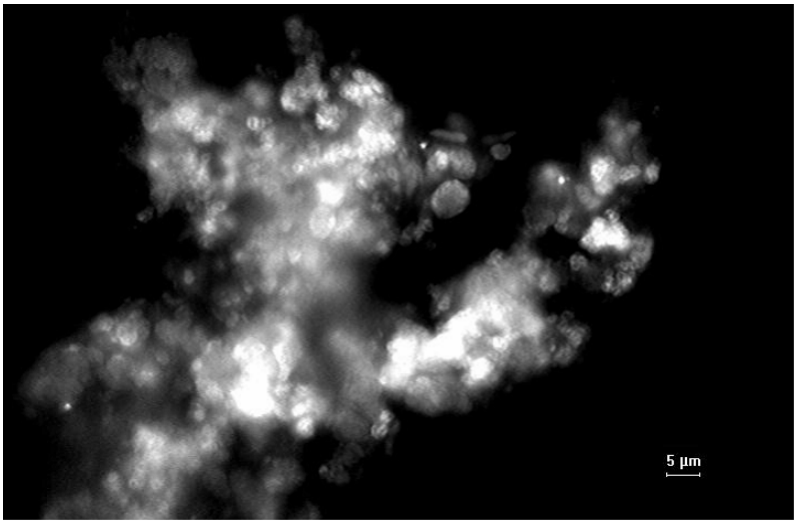

Figure 6: Epifluoresce micrograph of anaerobic ammonium-oxidizing enrichment reactor biomass after in situ hybridization with Amx820 Cy3-labeled probe.

CONCLUSIONS

In this work the difficulty to establish a conventional heterotrophic denitrification process to treat slaughterhouse wastewater was verified. More detailed studies to address a possible inhibition of denitrification by the effluent are being carried out in our laboratory. On the other hand, an unusual loss of ammonium nitrogen was observed in the denitrifying reactor. During the sludge enrichment period, providing optimal conditions for the ANAMMOX process, the consumption of nitrite and ammonium with the formation of N_2 was verified. Studies with fluorescence *in situ* hybridization concluded that microorganisms belonging to the phylum *Planctomycetes* were present in the enriched sludge. These results confirmed that nitrogen loss in the denitrifying reactor was by ANAMMOX. This process promises to be a sustainable treatment for nitrogen removal of the slaughterhouse effluent studied.

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