Inhibition effect of swine wastewater heavy metals and antibiotics on anammox activity
T. Lotti, M. Cordola, R. Kleerebezem, S. Caffaz, C. Lubello and M. C. M. van Loosdrecht

ABSTRACT
The feasibility of anaerobic ammonium oxidation (anammox) process to treat wastewaters containing antibiotics and heavy metals (such as the liquid fraction of the anaerobically digested swine manure) was studied in this work. The specific anammox activity (SAA) was evaluated by means of manometric batch tests. The effects of oxytetracycline, sulfathiazole, copper and zinc were studied. The experimental data of the short-term assays were fitted with an inhibition model to identify the half maximal inhibitory concentration (IC50). After 24 h exposures, IC50-values equal to 1.9, 3.9, 650 and 1,100 mg L⁻¹ were identified for copper, zinc, sulfathiazole and tetracycline respectively. The effect of prolonged exposure (14 days) to oxytetracycline and sulfathiazole was studied by means of repeated batch-assays. Anabolism and catabolism reactions were active during the inhibition tests indicating that anammox bacteria could grow even in the extreme conditions tested. Considering the average concentrations expected in swine wastewaters, the inhibitors studied do not seem to represent a problem for the application of the anammox process. However, in order to verify the effect of these compounds on the growth of anammox bacteria, continuous culture experiments could be conducted.

Key words | anammox, antibiotics, copper, inhibition, oxytetracycline, sulfathiazole, swine manure, zinc

INTRODUCTION
Anaerobic digestion is being widely implemented as an efficient process for removal of organic matter from manure allowing the recovery of energy in the form of biogas. Digester liquor after anaerobic digestion of manure contains high concentrations of nitrogen and phosphorus as well as heavy metals and pharmaceuticals fed to the livestock. Such wastewater needs to be treated before being discharged to receiving water bodies. Biological nitrogen removal is achieved mostly by complete oxidation to nitrate and subsequent reduction of the nitrate to dinitrogen gas under anoxic conditions at the expense of organic carbon (COD). However, COD in digested swine wastewater (after swine manure anaerobic digestion and solid/liquid separation) is not sufficient to remove nitrogen, and therefore external addition of COD is required for complete denitrification, which consequently leads to an increase in the cost of the operation.

Recently, a novel biological nitrogen removal process, anaerobic ammonium oxidation (anammox), which oxidizes ammonium to dinitrogen gas with nitrite as electron acceptor under strictly anaerobic conditions, has been introduced (Strous et al. 1998; van der Star et al. 2007). This process is advantageous over most commonly employed nitrification-denitrification processes as no external addition of COD and lower oxygen supply is required combined with a low sludge production. Therefore, manure digester effluent is expected to be a prime application of the anammox technique. Some investigations of the application of anaerobic digestion of swine slurry, following partial nitrification to oxidize ammonium to nitrite and anammox treatment have been reported (Hwang et al. 2005; Yamamoto et al. 2008; Molinuexo et al. 2009), but this treatment chain has not been yet applied in full scale.
Swine wastewater is a mixture of swine urine, excrement and service water and contains high concentrations of nitrogen as well as heavy metals, such as copper and zinc, and veterinary antibiotics. Due to the broad administration of these veterinary antibiotics, to prevent infections and treat diseases as well as growth promoters (Huang et al. 2001), swine wastewater is one of the most complicated agricultural wastewaters with respect to nitrogen removal. Oxytetracycline, belonging to the tetracycline class, is a common antibiotic with a broad range of activity and low cost. The molecules of the tetracycline class act by interfering with bacterial protein synthesis by binding to the 3OS ribosomal subunit and thus preventing the attachment of the transfer RNA (tRNA) and the subsequent amino acid chain elongation. Oxytetracycline is administered to livestock animals (including cattle, swine, poultry and fish) and can be found in manure (expressed as mg per kg of dry matter, mg kg DM⁻¹) at concentrations up to 136 mg kg DM⁻¹ (Winckler et al. 2005) and at average concentrations of 59 mg kg DM⁻¹ (Zhao et al. 2010). The concentration of oxytetracycline in swine wastewater was estimated as 232 mg L⁻¹ (Huang et al. 2001). Other most commonly found antibiotics in swine manure belong to the sulfonamide class including the cheap and widely used sulfathiazole (Huang et al. 2001). Sulfonamide antibiotics act as structural analogues and competitive antagonists of para-aminobenzoic acid (PABA), essential substrate with which the microorganisms synthesize folic acid. This, in turn, is an essential cofactor in the synthesis of amino acids and nitrogenous bases, precursors of nucleic acids. In individual manures peak concentrations up to 235 mg kg DM⁻¹ of antibiotics belonging to this class have been detected (Engels 2004). No appreciable sulfonamide antibiotic concentrations (less than 10 mg kg DM⁻¹) were found in 61 Chinese swine manures (Zhao et al. 2010). Huang et al. (2001) estimated the concentration of a chlortetracycline-sulfathiazole-penicillin mixture in swine wastewater as 1.4 g L⁻¹. Most antibiotics are excreted unmetabolized via swine manure (Mohring et al. 2009). It has been reported that as much as 30–90% of the antibiotics fed to cattle can be excreted unmetabolized (Sarmah et al. 2006). Although some researchers have studied the fate of antibiotics in specific environmental compartments, such as soil interstitial water or anaerobic lagoons (Kemper et al. 2008), and in different biological processes (Arikant et al. 2006), there is little information regarding the effect of antibiotics on anammox activity (van de Graaf et al. 1995; Fernández et al. 2009).

Copper and zinc are essential micronutrients for pig metabolism and their feed is supplemented with these elements, but most of the dietary supply is excreted. Consequently, the slurries contain high concentrations of copper and zinc (L’Herroux et al. 1997). Considering that during the activated sludge process the major portion of copper and zinc occurred in the solid fraction (Suzuki et al. 2010) and despite the fact that during the anaerobic digestion process most of the mineral elements are again present in the solid fraction (86% trapped within particles with a diameter between 3 and 25 μm, Marcato et al. 2008), a relevant concentration of these metals is still present in the digestate (about 1.5 mg L⁻¹ according to Vanotti et al. 2007).

The aim of this study was to evaluate the effect of elevated concentrations of veterinary antibiotics and heavy metals on the activity of anammox bacteria.

**MATERIALS AND METHODS**

**Specific anammox activity test**

Batch experiments were performed to evaluate the specific anammox activity (SAA) according to the procedure reported by Lotti et al. (2009). The assays were performed in closed bottles equipped with online manometric sensors (OxiTop Control System; WTW, OxiTop Control AN6, Weilheim, Germany). Each vessel (340 mL) had two lateral holes used to introduce substrate and inhibitor injections and supernatant sampling. The procedure is based on the measurement along time of the overpressure (hPa) generated by the nitrogen gas production (N₂) in closed vials. The dinitrogen gas production rate was calculated from the headspace overpressure measurements using the ideal gas law equation. The gas-phase composition was measured off-line on an Agilent 6890 at the end of a set of preliminary assays. N₂ was confirmed to account for the 98 ± 2% (v/v). NO and N₂O were below the detection limit. The percentage of activity that was maintained after the exposure to inhibitory compounds was calculated with respect to the average of the activities of the control assays (unexposed biomass). All tests were conducted in duplicate.

**Experimental set-up**

**Short-term tests**

The biomass was washed and re-suspended in a *washing medium*: a medium containing the microelements needed
to avoid nutrient limitation (van De Graaf et al. 1995) as well as 25 mM HEPES (N-2-hydroxyethyl-piperazine-N’-2-ethane sulfonic acid) buffer. The pH value of the medium was set to 7.5 with 0.1 M NaOH or H2SO4. The headspace and liquid phase (200 mL) was flushed with dinitrogen gas to guarantee anoxic conditions. The vials were placed in a thermostatic shaker, at 180 rpm and 30 °C. After headspace pressure stabilization was established, the pressure was reduced to the atmospheric level by inserting a needle connected to a water-filled bottle which acted as a water-lock; then substrates were added by spiked injections through the puncturable septum. The injected solutions were prepared with NaN3O2, (NH4)2SO4 and NH4HCO3 (bicarbonate was added to avoid inorganic-carbon limitation during the test) dissolved in high purity water obtained through a milli-QTM system. In the experiments performed the starting ammonium and nitrite concentration was 50 mg N L\(^{-1}\) (100 mg N L\(^{-1}\) as total nitrogen). Once the pressure reached a constant value (and all nitrite was assumed to be converted), a liquid sample was taken for chemical analysis. The inhibitory effect of copper, zinc, oxytetracycline and sulfathiazole was evaluated in duplicate.

**Long-term tests**

The experimental set-up of the long-term effect of exposure to oxytetracycline and sulfathiazole tests was the same as in the short-term tests described above, except for: (i) the number of manometric tests conducted, (ii) the addition of antibiotics at the end of each manometric test, (iii) an additional term in the calculation of the SAA:

(i) Eleven manometric tests were conducted once per day (5 days per week);
(ii) At the end of each manometric test a liquid sample (3 mL) was taken for chemical analysis as described for the short-term tests. The oxytetracycline and sulfathiazole amount (mg) withdrawn with this liquid sample were reintroduced in the vessel through injection of 3 mL of a solution appositely prepared (same as washing medium but with the addition of antibiotics). The liquid volume and composition was therefore about constant in each of the 11 tests;
(iii) In the calculation of the maximum SAA relative to successive injections, the biomass growth was taken into account considering that the nitrate produced was coupled with the carbon dioxide assimilated (0.254 C-mol NO3\(-1\)mol\(^{-1}\) according to Strous et al. 1998).

**Inoculum**

The granular anammox biomass used in the tests originated from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, The Netherlands (van der Star et al. 2007). The anaerobic digester supernatant treatment process consists of partial nitritation in a SHARON reactor, followed by anammox process performed in a 70 m\(^3\) internal circulation reactor. During the tests performed in presence of heavy metals, the initial biomass concentration in the vessels was 0.74 gVSS L\(^{-1}\) and its SAA was 0.68 ± 0.04 g N\(_2\)-N g VSS\(^{-1}\) d\(^{-1}\). When the effect of antibiotics was studied, the initial biomass concentration was 2.1 g VSS L\(^{-1}\) and its SAA was 0.69 ± 0.04 g N\(_2\)-N g VSS\(^{-1}\) d\(^{-1}\). The biomass was confirmed to consist of a ‘Brocadia’ enrichment during the period of the tests by fluorescence in situ hybridization (FISH), the sludge hybridized with AMX820 and not with KST157 probes (Schmid et al. 2001).

**Analytical methods**

Soluble nitrogen compounds were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem Methods 10-107-06-5-E for ammonium (range 0.1–10.0 mg N L\(^{-1}\), measurement of NH3 after increasing pH and evaporation) and 10-107-04-1-C for nitrate/nitrite (range 0.01–2.0 mg N L\(^{-1}\), direct measurement of nitrite, or measurement preceded by reduction of NO3\(^{-}\) to NO2\(^{-}\) to yield the concentration of NO3\(^{-}\) + NO2\(^{-}\)) according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.05 to 10 mg N L\(^{-1}\). Soluble copper (Cu\(^{2+}\)) and soluble zinc (Zn\(^{2+}\)) concentration in the liquid bulk were detected using commercial test kits according to the protocol of the manufacturer (brand: Dr. Lange test kits, Hach-Lange GmbH, Düsseldorf, DE, kits LCK329 and LCK529 for copper and LCK360 for zinc) and determined on a designated spectrophotometer (DR 2800). All the samples were filtered at 0.45 μm before analysis. Concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (APHA 2005).
Evaluation of IC\textsubscript{50}

The half maximal inhibitory concentration, IC\textsubscript{50}, is the concentration of the tested compound, which corresponds to 50% activity compared with a non-inhibited assay (control). The maximum specific removal rate experimental data were fitted with an inhibition model to identify the IC\textsubscript{50} (mg inhibitor L\textsuperscript{-1}/C\textsubscript{0}):

\[
\text{Rate} = \frac{\text{Rate}_{\text{max}}}{(1 + (I/\text{IC}_{50}))}
\]

where Rate is the specific N\textsubscript{2} production rate (g N\textsubscript{2}-N g VSS\textsuperscript{-1} d\textsuperscript{-1}) in the presence of inhibitors, Rate\textsubscript{max} is the maximum specific N\textsubscript{2} production rate and I is the concentration of the inhibitors (mg L\textsuperscript{-1}). The best fit was evaluated by the method of least squares.

RESULTS AND DISCUSSION

Short-term effects

Copper and zinc

Presence of increasing concentrations and prolonged exposure to copper and zinc leads to a decreasing specific anammox activity (SAA) (Figure 1). The major part of the inhibition occurs within 8 h exposure for both copper and zinc. In a rough evaluation both metals have a similar inhibitory effect. For copper there seemed to be a clear exposure effect (activity declined with increasing exposure time). For zinc such an effect was not clear from the results. In order to facilitate the comparison between the effects of the two metals investigated, the corresponding IC\textsubscript{50} after different exposure times were calculated (Table 1). The overall inhibitory potential was higher for copper than for zinc. After 24 h exposure at 1 mg L\textsuperscript{-1}, a realistic concentration for the anaerobic supernatant from pig manure digestion (Vanotti et al. 2007), the activity decrease was about 25 and 15% for copper and zinc respectively. The effect of exposure time was also strong for inhibition to copper as shown in Table 1. During the exposure to both metals however the observed stoichiometry was not affected, resulting in regular nitrate production (0.24 ± 0.04 mol nitrate produced per mol ammonium consumed), and therefore regular growth (Strous et al. 1998). Since in literature there is no information regarding heavy metals inhibition of anammox activity, the results presented in this study can be only compared with the results found for other microorganisms. Grunditz et al. (1998) reported zinc as a more significant inhibitor than copper for pure culture of nitrifying and nitrite reducing bacteria. Çeçen et al. (2010) showed a higher inhibition effect on nitrification for copper than for zinc in short term batch tests reporting an IC\textsubscript{50} of about 7 mg L\textsuperscript{-1} for both metals. Madoni et al. (1999) reported 49 and 22% inhibition on activated sludge oxygen uptake rate (OUR) and ammonium uptake rate (AUR) respectively, after 24 h exposure to 1 mg Cu L\textsuperscript{-1}, while 55 and 43% inhibition on OUR and AUR respectively were observed after 24 h exposition to 20 mg Zn L\textsuperscript{-1}. The fraction of copper and zinc adsorbed on the solid fraction and therefore not measured back in the liquid phase at the end of the 24 h exposure, was below 8% of the starting concentration without any evident difference between the two metals (Figure 2). The little amount adsorbed on biomass...
supports the validity of our results since the inhibition is related to the metal soluble fraction. Marcato et al. (2008) reported that most of the copper and zinc (86%) trapped in digested pig slurry was within particles between 3 and 25 μm and less than 2% was trapped within particles larger than 250 μm due to their low specific surface area. This agrees with the fact that the diameter of the granules employed in the experimentation was 1–2 mm.

**Table 1** | IC₅₀ for copper and zinc inhibitory effect on specific anammox activity as function of the exposure time

<table>
<thead>
<tr>
<th>Exposure duration [h]</th>
<th>1</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper IC₅₀ [mg Cu L⁻¹]</td>
<td>5.0</td>
<td>2.6</td>
<td>1.9</td>
</tr>
<tr>
<td>Zinc IC₅₀ [mg Zn L⁻¹]</td>
<td>4.5</td>
<td>4.2</td>
<td>3.9</td>
</tr>
</tbody>
</table>

**Figure 2** | Copper (squares) and zinc (circles) adsorbed on solid fraction during 24 h exposure as percentage of the starting concentration (mg L⁻¹).

**Figure 3** | Effect of oxytetracycline (left graph) and sulfathiazole (right graph) on the specific anammox activity expressed as percentage activity relative to the unexposed culture. Activity evaluated after exposure for 1 h (open diamonds), 8 h (close diamonds), 24 h (close circles) and 30 h (open circles).

**Oxytetracycline and sulfathiazole**

The effect of exposure to oxytetracycline and sulfathiazole on the SAA increased with higher concentrations and longer exposure time (Figure 3). In presence of oxytetracycline, during the first 8 h there was no appreciable inhibition, for longer exposures instead, the activity decreased. A higher concentration leads to decreasing activities. The inhibitory effect of sulfathiazole at concentrations higher than 250 mg L⁻¹ was already significant 2 h after the test started and increased continuously according to increasing exposure duration at concentrations higher than 100 mg L⁻¹. A negligible loss in activity was calculated at concentrations smaller than 250 mg L⁻¹ of both antibiotics. The estimated specific anammox activities showed that the inhibitory effects of sulfathiazole were higher than those resulting from oxytetracycline. The IC₅₀ evaluated after 24 h exposure was indeed smaller for sulfathiazole (650 mg L⁻¹) than for oxytetracycline (1,100 mg L⁻¹).

The inhibitory effect of tetracycline hydrochloride (an antibiotic belonging to the same class of oxytetracycline and similar to the latter in structure and properties) on anammox activity was reported having an IC₅₀ equal to about 210 mg L⁻¹, and this value also increased with exposure time (Fernández et al. 2009). The stronger effect reported by these authors could be related to the three times lower SAA of the biomass used (0.25 ± 0.01 g N₂-N g VSS⁻¹ L⁻¹). Campos et al. (2001) reported 50% activity inhibition at 250 mg L⁻¹ of oxytetracycline during continuous operation of a nitrifying bioreactor. The effect of this class of antibiotics is however very different according to the microorganism studied and results from literature are often contradictory. In anaerobic digestion and anaerobic lagoons
the methane production decrease was reported to be 27% at 3.1 mg oxytetracycline L\(^{-1}\) (Arikan et al. 2006), or negligible up to 250 mg oxytetracycline L\(^{-1}\) (Lallai et al. 2002), 42% at 25 mg L\(^{-1}\) of chlortetracycline (Loftin et al. 2005; the same author reported no inhibition effects in the case of oxytetracycline) and 45% at 10 mg L\(^{-1}\) of oxytetracycline and chlortetracycline together (Álvarez et al. 2010). The inhibitory effect of sulfathiazole on microorganisms applied for wastewater treatment is not extensively studied in literature, but a few useful results for comparison can be found. The methane production in anaerobic lagoons was reported to decrease of 14% at 25 mg sulfathiazole L\(^{-1}\) (Loftin et al. 2005). Baran et al. (2006) reported IC\(_{50}\) 16 mg sulfathiazole L\(^{-1}\) for the green alga Chlorella vulgaris.

### Long-term effect of exposure to oxytetracycline and sulfathiazole

The effect of prolonged exposition to oxytetracycline and sulfathiazole on the anammox activity was evaluated by repeated batch assays conducted for 14 days at regular intervals of 24 h (Figure 4). In the presence of 100 mg oxytetracycline L\(^{-1}\) the activity was about constant for the first 7 days while afterwards decreased to 75% of the control activity at the end of the experiment. At 500 mg oxytetracycline L\(^{-1}\) the activity decreased to 50% at day 14, a similar effect was reached after 24 h exposure to 1,000 mg oxytetracycline L\(^{-1}\) (Figure 3). A 40% decrease in activity was reported in the continuous operation of an anammox bioreactor after tetracycline hydrochloride was fed for 30 days at a concentration of 10 mg L\(^{-1}\) (Fernàndez et al. 2009). In the case of prolonged exposure of sulfathiazole a stronger inhibitory effect than oxytetracycline was observed. After 14 days in the presence of sulfathiazole the anammox activity decreased to 50% (at 100 mg L\(^{-1}\)) and 28% (at 500 mg L\(^{-1}\)) relative to the unexposed culture.

The nitrate produced (which is coupled to the carbon dioxide reduction for growth of anammox bacteria) during each batch test was calculated in order to evaluate the anabolic or anabolic and catabolic nature of the antibiotics’ inhibition. The production/consumption ratios between the nitrogen compounds involved in the anammox metabolism were also evaluated (nitrite on ammonium consumed and nitrate produced on ammonium consumed). No evident trend in nitrate production or remarkable discrepancy from the anammox stoichiometry reported by Strous et al. (1998) was found (data not shown). This indicates that both anabolism and catabolism reactions were active during the inhibition tests and therefore that, even at high antibiotic concentrations, anammox bacteria were still able to grow. CO\(_2\) uptake was however not directly measured.

### CONCLUSIONS

Presence of increasing concentrations and prolonged exposure to copper and zinc leads to a decreasing SAA. For copper, especially at concentrations higher than 2 mg Cu L\(^{-1}\), there seemed to be a clear exposure time effect (activity declined with increasing exposure time) while for zinc such an effect was not clear from the results. After 24 h exposure the evaluated IC\(_{50}\) were 1.9 and 3.9 mg L\(^{-1}\) for copper and zinc, respectively. The inhibiting effect of exposure to oxytetracycline and sulfathiazole on the SAA increased with higher concentrations and longer exposure
time. However, short-term exposure results showed a negligible loss of activity after 24 h exposure at concentrations up to 100 mg L\(^{-1}\) of oxytetracycline (IC\(_{50}\) = 1,100 mg L\(^{-1}\)) and sulfathiazole (IC\(_{50}\) = 650 mg L\(^{-1}\)). After 14 days exposure to 100 mg L\(^{-1}\) of oxytetracycline and sulfathiazole the anammox activity decreased to 75 and 50% relative to the unexposed culture, respectively. No evident trend in nitrate production nor remarkable discrepancy from the accepted anammox stoichiometry was found, indicating that both anabolism and catabolism reactions were active during the inhibition tests. Considering the average concentrations expected in swine wastewaters (after swine manure anaerobic digestion and solid/liquid separation), the inhibitors studied do not seem to represent a real hazard for the application of the anammox process with granular sludge, since a lower specific activity could be counterbalanced by higher biomass concentration in the reactor. However, continuous culture experiments could be conducted in order to verify the effect of these compounds on the growth of anammox bacteria.

**ACKNOWLEDGEMENTS**

Tommaso Lotti was supported by the Italian Ministry of Agriculture (project BRAIN, DM no. 16917/7503/10 issued on 23/07/2010). The authors wish to thank ‘Water-schap Hollandse Delta’ for the use of biomass from their full-scale anammox reactor.

**REFERENCES**


Marcato, C. E., Pinelli, E., Pouche, P., Winterton, P. & Guirresse, M. 2008 Particle size and metal distributions in...

First received 9 February 2012; accepted in revised form 9 May 2012

Downloaded from https://iwaponline.com/wst/article-pdf/66/7/1519/441819/1519.pdf by guest

by guest