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Anaerobic ammonium-oxidizing bacteria gain antibiotic resistance during long-term acclimatization

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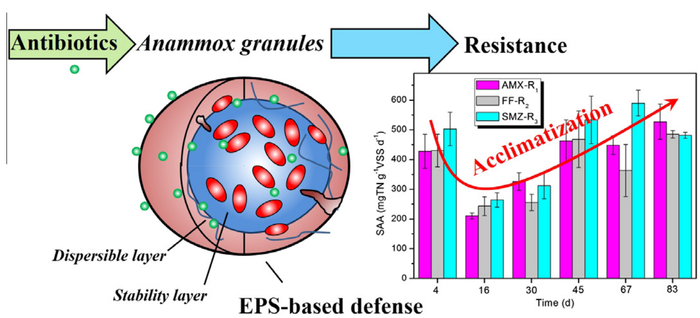
HIGHLIGHTS

- Amoxicillin, florfenicol and sulfamethazine had no acute toxicity on AnAOB.
- AnAOB showed antibiotic resistance during long-term acclimatization.
- AnAOB contained peptidoglycan-like components in their cell walls.
- The antibiotic resistance was primarily attributed to an EPS-based defense.

GRAPHICAL ABSTRACT



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ABSTRACT

Three broad-spectrum antibiotics, amoxicillin (AMX), florfenicol (FF) and sulfamethazine (SMZ), that inhibit bacteria via different target sites, were selected to evaluate the acute toxicity and long-term effects on anaerobic ammonium oxidation (anammox) granules. The specific anammox activity (SAA) levels reduced by approximately half within the first 3 days in the presence of antibiotics but no nitrite accumulation was observed in continuous-flow experiments. However, the SAA levels and heme c content gradually recovered as the antibiotic concentrations increased. Extracellular polymeric substances (EPS) analysis suggested that anaerobic ammonium-oxidizing bacteria gradually developed a better survival strategy during long-term acclimatization, which reduced the antibiotic stress via increased EPS secretion that provided a protective 'cocoon.' In terms of nitrogen removal efficiency, anammox granules could resist 60 mg-AMX L^{-1}, 10 mg-FF L^{-1} and 100 mg-SMZ L^{-1}. This study supported the feasibility of using anammox granules to treat antibiotic-containing wastewater.

1. Introduction

The anaerobic ammonium oxidation (anammox) reaction is catalyzed by chemolithoautotrophic bacteria belonging to the *Planctomycetes* phylum that use ammonium as an electron donor and nitrite as an electron acceptor (van de Graaf et al., 1995). These anaerobic ammonium-oxidizing bacteria (AnAOB) contribute significantly to the release of fixed nitrogen back to the atmosphere and are applied in wastewater treatment for an environmentally friendly and cost-effective method of nitrogen removal (van Loosdrecht and Brdjanovic, 2014). To date, anammox-based processes constitute a robust and reliable process for treating wastewater with high nitrogen concentrations in mesophilic conditions, and over 100 full-scale plants have been installed worldwide (Lackner et al., 2014). A majority

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(approximately 75%) of installations are applied toward the side-stream treatment of municipal wastewater. With recent advances in main-stream treatment, a net energy-producing sewage treatment plant providing effective nutrient removal is nearing feasibility (Lotti et al., 2014a,b; van Loosdrecht and Brdjanovic, 2014).

Due to the slow growth rates, low cell yields and highly variable responses to external cues, the application and industrialization of anammox-based processes has been restricted by the inhibitory substances that are common in nitrogen-rich wastewater (Jin et al., 2012). For example, antibiotics originated from anthropogenic input from wastewater discharge, manure disposal, and aquaculture, frequently exist in pharmaceutical wastewater, swine wastewater, black water and municipal sludge (Kümmerer, 2009; Michael et al., 2013), which are characterized by high chemical oxygen demand (COD) and high levels of ammonium species. These organic pollutants have been efficiently removed by an anaerobic methanogenic process that also enabled the recovery of energy in the form of biogas; additionally, the ammonium species have been treated with physicochemical and conventional biological methods (Tang et al., 2011a). However, the COD of the digested liquid is not sufficient to remove nitrogen. Therefore, the addition of external organic matter is required for complete denitrification; this requirement increases the operation costs (Lotti et al., 2012). Recently, partial nitritation–anammox (PN–anammox) processes have been considered as promising alternatives for biological nitrogen removal in these wastewater with the potential, in combination with anaerobic digestion, to turn the energy balance of wastewater treatment neutral or even positive (Fernández et al., 2009; van Loosdrecht and Brdjanovic, 2014). However, a few antibiotics were not completely degraded during the anaerobic digestion process and were found in the effluent (Michael et al., 2013). Most works have generally agreed that the observed effects on the process performance were not critical for digestion efficiency (Chelliapan et al., 2006; Oktem et al., 2008). The significant inhibition induced by lincomycin and different effects on nitrification in the presence of chloramphenicol and oxytetracycline have been previously documented (Luis Campos et al., 2001; Oktem et al., 2008). Nevertheless, few studies have investigated the behavior of anammox granules in the presence of antibiotics (Fernández et al., 2009; Hu et al., 2013; van de Graaf et al., 1995; Yang et al., 2013; Zhang et al., 2014).

In this study, three widely used, broad-spectrum antibiotics (i.e., amoxicillin (AMX), florfenicol (FF) and sulfamethazine (SMZ)) that act on different target sites of bacterial cell, were used to evaluate their acute toxicities and long-term effects on anammox granules. In addition, the effects of long-term acclimatization of AnAOB to these antibiotics were highlighted. Moreover, the role of extracellular polymeric substances (EPS) in antibiotic resistance was clarified.

2. Methods

2.1. Synthetic wastewater and inoculums

Inorganic synthetic wastewater that contained substrates, bicarbonate and trace elements (similar to the influent described by Yang and Jin, 2013) were introduced to a reactor. Equimolar amounts of ammonium and nitrite in the forms of $(NH_4)_2SO_4$ and $NaNO_2$, respectively, were supplied as needed.

Anammox seed granules were harvested from a high-loaded laboratory-scale up-flow anaerobic sludge blanket (UASB) reactor (R_0) fed with synthetic medium at a stable nitrogen removal rate (NRR) of approximately 10 kgN m^{-3} d^{-1}. These mature anammox granules possessed excellent settleability (over 70 m h^{-1}) and superior granule diameter (5.1 ± 1.6 mm in average), favoring biomass retention. The sludge was dominated by AnAOB of the genus *Candidatus Kuenenia stuttgartiensis*.

2.2. Acute toxicity assays

Batch exposure assays were performed in serum flasks with 160 mL total volume and 120 mL liquid phase volume. Equimolar amounts of ammonium and nitrite were added to the mineral medium for final ammonium or nitrite concentration of 100 mg N L^{-1} each. A total of 100 mL of basal mineral medium and 1.25 mL of trace element solutions I and II per liter of sterilized water (which had a composition similar to that of the synthetic wastewater) were introduced. Then, each antibiotic was added to the serum flasks as needed, along with 10 mL of anammox biomass. The volatile suspended solid (VSS) concentration was approximately 2.5 g L^{-1} in each serum flask. The initial pH was fixed at approximately 7.5 by injections of 1 M hydrochloric acid or sodium hydroxide. The serum vials were subsequently flushed with 99.99% pure argon for 10 min and immediately sealed with butyl rubber to avoid oxygen leakage. Then, the serum flasks were placed in a 35 ± 1 °C thermostatic shaker at 180 rpm. The specific anammox activity (SAA) determination and calculation methods were consistent with those described by Yang and Jin (2013). The antibiotic concentrations tested were 0, 5, 20, 50, 100, 200, 500 and 1000 mg L^{-1} for AMX; 0, 2, 4, 8, 16, 32, 64, 96 and 128 mg L^{-1} for FF; and 0, 5, 20, 40, 80, 160, 240 and 320 mg L^{-1} for SMZ.

2.3. Anammox reactors and operational strategy

To investigate the long-term effects of AMX, FF and SMZ on the anammox process, three high-loaded UASB reactors (AMX-R_1, FF-R_2, and SMZ-R_3) with effective volumes of 1.0 L each were fabricated from Plexiglas, covered with black cloth to prevent light-related inhibition and then placed in a thermostatic room at 35 ± 1 °C. The continuous-flow experiments featured a constant influent substrate level of 280 mg L^{-1}. Given that these antibiotics are almost growth inhibitors, the high biomass concentration is a prerequisite for treating antibiotic-containing wastewater. After inoculation, the initial VSS and suspended solid (SS) concentrations in the three reactors were approximately 16.7 and 25.0 g L^{-1}, respectively. Due to the overuse of antibiotics in livestock feed and the incomplete metabolism of livestock, the peak concentrations of oxytetracycline, chlortetracycline and sulfonamide antibiotics in manure samples have been reported as hundreds of milligrams per liter (Álvarez et al., 2010; Lotti et al., 2012). The concentration of chlortetracycline–sulfathiazole–penicillin mixture in swine wastewater was estimated as 1.4 g L^{-1} (Lotti et al., 2012). In view of the incomplete degradation and the long half-life of these antibiotics during the anaerobic digestion process (Arikan et al., 2006), considerable amounts of antibiotics might be introduced to anammox-based reactors. Thus, the setting of influent antibiotic concentrations in this study intends to evaluate the potential inhibition of antibiotics on anammox process and the corresponding specific biomass load equivalents are listed in Table 1. When the antibiotics were added, the hydraulic retention time (HRT) was maintained at 1.2 h. In this study, the biomass was lost mainly through sampling and washout (~20 mgSS L^{-1}), thus the sludge retention time (SRT) was calculated approximately as 60 d.

2.4. Analytical procedures

The ammonium, nitrite, nitrate, VSS and suspended solid concentrations as well as the pH and settling velocity (V_S) were