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Anammox biofilm in activated sludge swine wastewater treatment plants

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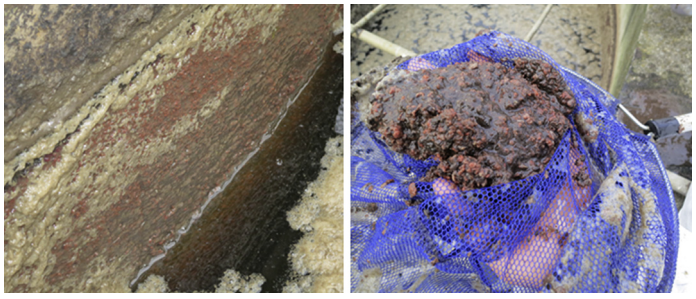
HIGHLIGHTS

- Red biofilm (BF) was found in 3 AS treatment facilities for swine wastewater (WW).
- The BF showed high anammox (AMX) copy numbers and activities.
- Their highest values were 1.35×10^{12} copies/g-VSS and 295 μmoL/g-VSS/h.
- The BF included *Planctomycetes* (max. 17.7%, comparable to enriched AMX sludge).

GRAPHICAL ABSTRACT



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ABSTRACT

We investigated anammox with a focus on biofilm in 10 wastewater treatment plants (WWTPs) that use activated sludge treatment of swine wastewater. In three plants, we found red biofilms in aeration tanks or final sedimentation tanks. The biofilm had higher anammox *16S rRNA* gene copy numbers (up to 1.35×10^{12} copies/g-VSS) and higher anammox activity (up to 295 μmoL/g-ignition loss/h) than suspended solids in the same tank. Pyrosequencing analysis revealed that *Planctomycetes* accounted for up to 17.7% of total reads in the biofilm. Most of them were related to *Candidatus* Brocadia or *Ca.* Jettenia. The highest copy number and the highest proportion of *Planctomycetes* were comparable to those of enriched anammox sludge. Thus, swine WWTPs that use activated sludge treatment can fortuitously acquire anammox biofilm. Thus, concentrated anammox can be detected by focusing on red biofilm.

1. Introduction

Activated sludge treatment is the most common biological wastewater treatment process, which is used to treat sewage,

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night soil wastewater, landfill leachate, and industrial wastewater. Most swine farms in Japan use it to treat swine wastewater, which is a mixture of urine, feces, and service water. Raw swine wastewater contains high concentration of biochemical oxygen demand (BOD) (e.g., 8700 ± 5200 mg L^{-1}, mean ± SD) and nitrogen (e.g., 2500 ± 1300 mg L^{-1}) (Waki et al., 2010a). Therefore, it is treated for a long hydraulic retention time ranging from days to weeks. After treatment, although BOD becomes much lower (e.g., 59 ± 83 mg L^{-1}), the concentration of nitrogen remains high (e.g., 430 ± 540 mg L^{-1}) (Waki et al., 2010a). Nitrogen can be removed by nitrification followed by denitrification using organic compounds in the wastewater as electron donors, but the BOD concentration in raw wastewater is insufficient to remove nitrogen.

In contrast to nitrification and denitrification, anaerobic ammonium oxidation (anammox) oxidizes NH_4^+ to N_2 with NO_2^- as an electron acceptor under strictly anaerobic conditions (Mulder et al., 1995; Strous et al., 1997). This process does not consume BOD to metabolize nitrogen, and therefore can remove nitrogen from low-BOD wastewater.

Anammox was discovered in a wastewater treatment plant (WWTP) treating wastewater from a baker's yeast production plant (Mulder et al., 1995). Since then, it has been found in many ecosystems, including seas, rivers, lakes, paddy fields, and wetlands (Waki et al., 2015; Zhu et al., 2010, 2011), and in conventional WWTPs (Tsushima et al., 2007). However, it is not dominant in most cases. Since the growth rate of anammox organisms is very slow, with a doubling time of >1 week (Strous et al., 1998; Yasuda et al., 2011), it is uncommon to find high concentrations. Therefore, anammox enrichment using seed sludge taken from those ecosystems or from conventional WWTPs can take months to years. Hence, the preparation of anammox-enriched sludge is the most time-consuming step in starting up anammox reactors. Acquiring sludge containing high concentration of anammox is indispensable to shorten the anammox reactor start-up process.

In municipal activated-sludge WWTPs, anammox was detected broadly by quantitative real-time polymerase chain reaction (qPCR) analysis (Tsushima et al., 2007), and the maximum contribution to nitrogen removal was 7.26% (Wang et al., 2015). Anammox organisms were enriched from the sludge taken from the WWTP over several months by incubation in inorganic artificial wastewater (Chamchoi and Nitisoravut, 2007; Sun et al., 2011). When the anammox biomass concentration in sludge becomes high, the sludge appears red (Rouse et al., 2003; van de Graaf et al., 1996; van der Star et al., 2008), but environmental and WWTP samples in which anammox is detected are not usually red. However, in an activated sludge WWTP in Taiwan that treats landfill leachate, a red anammox-rich biofilm was discovered (Wang et al., 2010), with a maximum contribution to nitrogen removal of 68%. Such enriched anammox sludge could be beneficial for the improvement of nitrogen removal at the WWTP and for the start-up of other anammox reactors.

In activated sludge WWTPs that treat swine wastewater, we detected anammox by a tracer method at half of the farms surveyed (Waki et al., 2010b). Anammox activity was detected in biofilm at three orders of magnitude higher than in suspended sludge. Thus, biofilms in conventional activated-sludge WWTPs might have high concentrations of anammox.

Here, we investigated anammox biomass in activated sludge WWTPs that treat swine wastewater, with a focus on biofilm. In three WWTPs, we found red biofilms in aeration tanks or sedimentation tanks. The presence of anammox was confirmed by qPCR and by incubation with a ^{15}N tracer. We analyzed the microbial community of the biofilm by pyrosequencing.

2. Materials and methods

2.1. Samples

We investigated anammox biofilm at 10 swine farms (A–J) in Ibaraki and Shizuoka prefectures, Japan (Table 1). Farms A–H were newly selected, and farms I and J were included because biofilms were observed at these farms in a preliminary investigation. We searched for biofilms on the walls of activated sludge aeration tanks and sedimentation tanks or perceived it by scraping with a 2-mm sieve. We collected the biofilms that we detected. We also collected the sample of wastewater containing suspended solids (SS) from the aeration tank at each farm. The biofilm samples were ground in a mortar, and SS samples were collected by centrifugation (8000–14 000×*g*); the samples were then analyzed for anammox and denitrification activity and by qPCR.

2.2. Real-time qPCR analysis of anammox bacteria

Total DNA was extracted twice from each sample by using a FastDNA SPIN Kit for Soil (MP Bio, Tokyo, Japan), bulked, and purified with a QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). The abundance of anammox bacterial 16S rRNA genes was determined in triplicate by qPCR amplification using SsoAdvanced SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). qPCR was performed in 96-well optical plates in a real-time PCR detection system (CFX96 or MyiQ2, Bio-Rad) with the anammox-specific primer sets S-*-Amx-0368-a-A-18 and AMX820 (Schmid et al., 2005). Each reaction mixture (20 μL) contained 10 μL of SsoAdvanced SYBR Green Supermix, 5 ng of total DNA, and 6 pmol of each primer. The following program was used: 98 °C for 2 min, followed by 40 cycles of denaturation at 98 °C for 5 s and annealing and extension at 58 °C for 60 s. The specificity of amplification was determined by the melting curve. Copy numbers were determined by comparison with standard curves constructed using 10-fold serial dilutions (10^1–10^6 gene copies·μL^{-1}) of plasmids containing corresponding gene fragments. Coefficients of determination (r^2) were >0.95 for all standard curves.

2.3. Pyrosequencing analysis

Pyrosequencing of 16S rRNA genes was conducted with samples H (SS and biofilm, Nov. 2014), I-3 (biofilm, Nov. 2014), and J-3 (biofilm, Oct. 2014), with PCR products (239 bp) amplified with the forward primer 563 F and four mixed reverse primers, R1–4 (Zhang et al., 2012). Each amplification reaction mixture (50 μL) contained 5 ng of total DNA, 1.25 U AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA), 5 μL of 10 × Gold buffer, 0.2 mM dNTPs, 2 mM $MgCl_2$, and 25 pmol of each primer. PCR amplification used the following program: 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were purified with a QIAquick PCR purification kit (Qiagen). The adaptor was ligated using an Ion Xpress Plus Fragment Library Kit (Life Technologies, Carlsbad, CA, USA) and sequenced using an Ion PGM sequencing kit on an Ion PGM system (Thermo Fisher Scientific, Waltham, MA, USA). Reads of <150 bp raw data were removed, and bases corresponding to the primers and with low-quality scores were trimmed using Btrim software with the default cut-off point (Kong, 2011). Chimeric sequences were detected by Chimera Slayer software (Haas et al., 2011) and eliminated. Operational taxonomic units were selected and assigned in QIIME software (Caporaso et al., 2010). All trimmed raw reads of ≥150 bp have been deposited in the DDBJ database under accession number DRA004785.