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Anaerobic ammonium oxidation in traditional municipal wastewater treatment plants with low-strength ammonium loading: Widespread but overlooked

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ABSTRACT

Occurrence of anaerobic ammonium oxidation (anammox) in marine and freshwater systems has greatly changed our understanding of global nitrogen (N) cycle and promoted the investigation of the role and ecological features of anammox in anthropogenic ecosystems. This study focused on the spatio-temporal abundance, activity, and biodiversity of anammox bacteria in full-scale municipal wastewater treatment plants (WWTPs) via traditional nitrification/denitrification route with low-strength ammonium loading. The anammox bacteria were detected in all the treatment units at the five WWTPs tested, even in aerobic zones (dissolved oxygen >2 mg L^{-1}) with abundance of 10^5–10^7 hydrazine synthase (*hzs*) gene copies g^{-1}. The ^{15}N-isotope tracing technology revealed that the anammox rates in WWTPs ranged from 0.08 to 0.36 μmol N g^{-1} h^{-1} in winter and 0.12–1.20 μmol N g^{-1} h^{-1} in summer with contributions of 2.05–6.86% and 1.71–7.26% to N_2 production, respectively. The diversity of anammox bacteria in WWTPs was distributed over only two genera, *Brocadia* and *Kuenenia*. Additionally, the exploration of potential interspecies relationships indicated that ammonia oxidation bacteria (AOB) was the major nitrite-substrate producer for anammox during nitrification, while *Nitrospira*, a nitrite oxidation bacteria (NOB), was the potential major competitor for nitrite. These results suggested the contribution of N-removal by the widespread of anammox has been overlooked in traditional municipal WWTPs, and the ecological habitats of anammox bacteria in anthropogenic ecosystems are much more abundant than previously assumed.

1. Introduction

Biogeochemical nitrogen (N) cycle is primarily driven by diverse microorganisms (Falkowski et al., 2008). Denitrification by heterotrophic bacteria has been regarded as the only pathway for the loss of fixed nitrogen to the atmosphere (Burgin and Hamilton, 2007). This concept has been challenged by the discovery of anaerobic ammonium oxidation (anammox) mediated by autotrophic anammox bacteria capable of oxidizing ammonia directly to nitrogen gas (N_2) without emission of nitrous oxide (N_2O) (Kuypers et al., 2003; Dalsgaard et al., 2003; Kartal et al., 2011).

Anammox has been detected in many natural ecosystems worldwide. In marine systems, the anammox is ubiquitous at the hotspots occurring in oxygen minimum zones (OMZ) (Dalsgaard et al., 2003; Ward et al., 2009; Pitcher et al., 2011; Hannig et al., 2007; Lam et al., 2007), and has been found to be responsible for up to 50% of marine N loss (Thamdrup and Dalsgaard, 2002; Arrigo, 2005). Anammox have also been detected ubiquitously in freshwater systems at all levels of substrate concentration (Zhu et al., 2013; Schubert et al., 2006; Wang et al., 2012a; Zhang et al., 2007), and neither temperature nor substrate-loading affected anammox occurrence (Zhu et al., 2013). These findings indicate that anammox may have a wide environmental habitat, and be

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responsible for a significant proportion of N_2 production from natural ecosystems.

In addition, anammox processes have been detected in anthropogenic ecosystems, such as constructed wetlands (Zhu et al., 2011a), plant soil (Zhu et al., 2011b; Hu et al., 2011) and recirculation aquaculture systems (van Kessel et al., 2010; Tal et al., 2006). As the largest biotechnological application worldwide, municipal wastewater treatment plants (WWTPs) have played a great role in the N cycle. However, these systems contributed to 1.3% of the total Nitrous oxide (N_2O) emitted (IPCC, 2001). Nevertheless, it is still not clear whether anammox occurrence is widespread in conventional municipal WWTPs, which could be significant for understanding the global N cycle balance and N-flux calculation model.

Based on previous studies (Zhu et al., 2013, 2011a, 2011b; Wang et al., 2012a; Zhu et al., 2011a, Zhu et al., 2011b), a new hypothesis was raised in this study, that is anammox occurs in conventional municipal WWTPs with low-ammonium loading. Although the anammox process has been widely used in high-strength (500–1500 mg NH_4^+–N L^{-1}) wastewater treatment (Lackner et al., 2014), it has been verified that substantial anammox activity could be maintained with reclaimed water (ammonia<2 mg L^{-1}) and undesirable N_2O emissions could be reduced (Zhu et al., 2011a). Therefore, the distribution, contribution and microbial mechanism of anammox in municipal WWTPs were investigated in this study. Specifically, a total of 12 treatment units in five municipal WWTPs at the Northern China were investigated for the occurrence of anammox using molecular and ^{15}N isotope tracing techniques. The results were compared in terms of treatment processes, treatment capacities, operation modes and seasonal variations. In addition, the interspecies relationships among anammox bacteria and nitrifiers (ammonia oxidation bacteria (AOB) and archaea (AOA), nitrite oxidation bacteria (NOB)) were analyzed. The ubiquitous nature and substantial contribution of anammox processes to conventional municipal WWTPs were verified for the first time.

2. Material and methods

2.1. Municipal WWTPs and samples

A total of twenty-four seasonal samples (12 samples in summer and 12 samples in winter) were collected from all 12 treatment units at five municipal WWTPs that employ different treatment processes including anaerobic/anoxic/oxic process (AAO) at the Xiaohongmen WWTP, anaerobic/anoxic/oxic process (AAO) at the Gaobeidian WWTP, oxidation ditch process (OD) at the Jiuxianqiao WWTP, a membrane bio-reactor (MBR) at the Beixiaohe WWTP, and a sequencing batch reactor (SBR) at the Wujiacun WWTP. Detailed information regarding the long-term operation of these five WWTPs, including flow rate, suspended solids (ss), sludge retention time (SRT), hydraulic retention time (HRT), temperature (T), dissolved oxygen (DO), and pH, are listed in the Table S1. The samples were collected at the treatment units at least in triplicate, and transported to the laboratory on ice. A portion of each sample was used to determine the anammox activity, while the remainder was used for chemical analyses and subsamples were stored at −80 °C for later DNA extraction and molecular analysis.

2.2. Chemical analyses

The ammonium (NH_4^+–N), nitrite (NO_2^-–N) nitrate (NO_3^-–N) and total nitrogen (TN) contents were measured using an Automated Ion Analyzer FIA (QuickChem 8500, Lachat Instruments, Milwaukee, WI, USA). The dissolved oxygen (DO), pH and temperature (T) were monitored *in-situ* using a WTW oxi/340i oxygen probe (WTW Company, Weilheim, Germany). The chemical oxygen demand (COD) and mixed liquid volatile suspended solids (MLVSS) were determined according to the standard methods (APHA et al., 1995). The detailed sample characteristics of the treatment units at five municipal WWTPs are listed in Table S2.

2.3. Measurement of anammox and denitrification rate with ^{15}N isotopic tracing technique

Mixed sludge-liquid samples of municipal WWTPs samples were added to He-flushed, 12.6 mL glass vials (Exetainer, Labco, High Wycombe, Buckinghamshire, UK), after which they were mixed with N_2-purged *in-situ* wastewater and pre-incubated at ambient temperature (summer (25 ± 0.9 °C) and winter (13 ± 1.3 °C), except for those from digested sludge that were incubated at 30 ± 1.1 °C in summer and 24 ± 0.7 °C in winter) for 24–36 h to deplete residual NO_x^- and oxygen. Subsequently, 100 μl of N_2-purged stock solution of each isotopic mixture ((i) $^{15}NH_4^+$ (>99.6% ^{15}N), (ii) $^{15}NH_4^+ + {}^{14}NO_3^-$ and (iii) $^{14}NH_4^+ + {}^{15}NO_3^-$ (>99.0% ^{15}N)) was injected through the septa of each vial to reach a final concentration of 50 mg L^{-1} close to the *in-situ* TN concentration of 50–65 mg L^{-1} (Zhu et al., 2013, 2011a, 2011b). Incubation of the samples at ambient temperatures was interrupted at various time points (0 h, 4 h, 8 h, 16 h, and 32 h) by injecting 200 μL of 7 M $ZnCl_2$ solution to inhibit the biological activity in the Exetainer. The evolution of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ in nitrification, denitrification and anammox processes involving labeled/unlabelled NH_4^+ and NO_x^- was shown in Fig. 1 and Fig. S1. The potential contribution of anammox and denitrification to N_2 production (or N removal) were then calculated based on the output $^{29}N_2$ and $^{30}N_2$, which were measured by the continuous flow isotope ratio mass spectrometry using a MAT 253 system with Gasbench II and an autosampler (Bremen, Thermo Electron Corporation, Finnigan, Germany) (Thamdrup and Dalsgaard, 2002). The ratio of anammox role (*Ra* %) was calculated by the potential contribution *via* anammox to total N removal (anammox plus denitrification).

2.4. DNA extraction, PCR, cloning, sequencing, and phylogenetic analysis

Genomic DNA was extracted from 0.2 g MLVSS of each dry samples using a FastDNA® SPIN Kit for Soil (QBIOgene Inc., Carlsbad, CA, USA) with a beating time of 45 s and a speed of 5.5. PCR was conducted to detect anammox 16S rRNA genes listed in Table S3, after which the purified PCR products were ligated and cloned using the pGEM®-T Easy system (Promega, Madison, WI, USA). One hundred clones were picked from each of the PCR products. The inserts were analyzed using T7 and SP6 vector primers and the positive amplicons were analyzed by restriction endonucleases *Hae* III and *Hha* I (Promega, Madison, WI, USA). Clones of representative digestion patterns were selected for sequencing and deposited in GenBank (KM609124–KM609183). All sequences and their relatives obtained from an NCBI-BLAST search of the GenBank database were then aligned using Clustal X 1.83 (Thompson et al., 1997). The phylogenetic trees were constructed by neighbor-joining (NJ) with the Jukes-Cantor correction using the MEGA 4.0 package (Tamura et al., 2007).

2.5. Quantitative PCR assay

The abundance of anammox bacteria, nitrifiers (AOA, AOB, and NOB) and total bacteria were determined using a Stratagene Mx3005p QPCR system (Agilent Technologies, USA) with the SYBR-Green approach (TAKARA, Dalian, China). The hydrazine synthase (*hzs*) function gene of anammox, *amo*A function gene of AOA and