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Source identification of nitrous oxide emission pathways from a single-stage nitritation-anammox granular reactor

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ABSTRACT

Nitrous oxide (N_2O) production pathway in a signal-stage nitritation-anammox sequencing batch reactor (SBR) was investigated based on a multilateral approach including real-time N_2O monitoring, N_2O isotopic composition analysis, and *in-situ* analyses of spatial distribution of N_2O production rate and microbial populations in granular biomass. N_2O emission rate was high in the initial phase of the operation cycle and gradually decreased with decreasing NH_4^+ concentration. The average emission of N_2O was $0.98 \pm 0.42\%$ and $1.35 \pm 0.72\%$ of the incoming nitrogen load and removed nitrogen, respectively. The N_2O isotopic composition analysis revealed that N_2O was produced via NH_2OH oxidation and NO_2^- reduction pathways equally, although there is an unknown influence from N_2O reduction and/or anammox N_2O production. However, the N_2O isotopomer analysis could not discriminate the relative contribution of nitrifier denitrification and heterotrophic denitrification in the NO_2^- reduction pathway. Various *in-situ* techniques (*e.g.* microsensor measurements and FISH (fluorescent *in-situ* hybridization) analysis) were therefore applied to further identify N_2O producers. Microsensor measurements revealed that approximately 70% of N_2O was produced in the oxic surface zone, where nitrifiers were predominantly localized. Thus, NH_2OH oxidation and NO_2 reduction by nitrifiers (nitrifier-denitrification) could be responsible for the N_2O production in the oxic zone. The rest of N_2O (ca. 30%) was produced in the anammox bacteria-dominated anoxic zone, probably suggesting that NO_2^- reduction by coexisting putative heterotrophic denitrifiers and some other unknown pathway(s) including the possibility of anammox process account for the anaerobic N_2O production. Further study is required to identify the anaerobic N_2O production pathways. Our multilateral approach can be useful to quantitatively examine the relative contributions of N_2O production pathways. Good understanding of the key N_2O production pathways is essential to establish a strategy to mitigate N_2O emission from biological nitrogen removal processes.

1. Introduction

Nitrous oxide (N_2O) emission from wastewaters is a growing concern and accounts for about 6% of N_2O global emission from all sources (the sixth largest contributor) (Ciais et al., 2013). During wastewater treatment, N_2O is mainly released from biological nitrogen removal systems. Large variations in the N_2O emissions were reported from biological nitrogen removal processes at bench-scale (0–95% of nitrogen load) and full-scale wastewater treatment plants (0–14.6% of nitrogen load) (Kampschreur et al., 2009b). Conventionally, nitrification and denitrification (N&DN) process was employed for nitrogen removal from wastewaters.

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However, anaerobic ammonium oxidation (anammox) process has potential to transform nitrogen removal from wastewaters. Nitritation and anammox processes have been used as an alternative treatment process for NH_4^+-rich wastewater streams (such as digester liquor) and considered as more economical and environmental friendly due to lower oxygen requirement, no external carbon source demand and less sludge production (Kuenen, 2008; Mulder et al., 1995; Okabe et al., 2011a). There are presently more than 110 full-scale anammox-based treatment plants worldwide and about 90% of the plants are being operated as single-stage systems (nitritation and anammox in one reactor) (Ali and Okabe, 2015; Lackner et al., 2014).

Extensive studies were conducted to study N_2O emission dynamics and patterns from both two-stage and single-stage nitritation-anammox processes (Table 1). There are three scientifically known biological pathways of N_2O production in wastewater treatment systems: hydroxylamine (NH_2OH) oxidation during nitrification ($NH_4^+ \rightarrow NH_2OH \rightarrow NOH \rightarrow N_2O$) (Law et al., 2012; Wunderlin et al., 2012) and NO_2^- reduction by nitrifiers (nitrifier-denitrification) (Colliver and Stephenson, 2000; Wrage et al., 2001) and by denitrifiers (heterotrophic denitrification) (Lu and Chandran, 2010). Though not conclusive, possibility of low-level N_2O production by anammox bacteria was also stated previously by some researchers (Harris et al., 2015; Lotti et al., 2014). Although relatively low N_2O productions (averagely 0.27% of the incoming nitrogen load) were reported for anammox reactors (Desloover et al., 2012; Kampschreur et al., 2008; Okabe et al., 2011a), it was confirmed that anammox reaction was not a main source of the N_2O emission (Okabe et al., 2011a). For two-stage nitritation-anammox processes, it was reported that NH_2OH oxidation pathway accounted for approximately 65% of total N_2O production from partial nitrification (PN) reactor fed with NH_4^+-rich inorganic synthetic wastewater (Rathnayake et al., 2013). The source of N_2O was different when a PN reactor was fed with NH_4^+-rich organic synthetic wastewater (Ishii et al., 2014). In their study, heterotrophic denitrification was a main source of N_2O emission from the PN reactor (70–80% of total N_2O production). For single-stage nitritation-anammox processes, the information related to N_2O production pathways is very limited. The major pathways of N_2O production in a pilot-scale PN-anammox sequencing batch reactor (SBR) were investigated using online isotopic analysis of off-gas N_2O with quantum cascade laser absorption spectroscopy (QCLAS) (Harris et al., 2015). In this study, N_2O emissions increased at high DO concentrations and it was concluded that this increase in N_2O was due to enhanced nitrifier denitrification based on N_2O isotopic site preference (SP) measurement. However, the SP value was much higher (up to 40‰) than previously reported in wastewater treatment systems, though higher SP values (up to 40‰) were observed in soil environments (Toyoda et al., 2011b). Therefore, more studies are needed to fully understand N_2O production pathways in single-stage nitritation-anammox systems.

In order to identify the sources of N_2O production in a single-stage nitritation-anammox process, a lab-scale sequential batch reactor (SBR) was operated and N_2O production was monitored at a reactor and microbial community (i.e., granule) level. Microbial community structure was analyzed based on 16S rRNA gene sequences using next-generation sequencing (Illumina MiSeq), and then the spatial distribution and abundance of important N-cycle stakeholders, ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and anammox bacteria, in granules were analyzed by fluorescence *in-situ* hybridization (FISH). The microbial community distribution was related to *in-situ* N_2O production as measured by microsensors. Furthermore, temporal changes in intermolecular ^{15}N-site preference (SP) of N_2O in off-gas were measured along with temporal changes in NH_4^+, NO_2^-, NO_3^-, DO and pH during reactor operational cycles. Finally, all data were integrated to identify the source of produced N_2O in the single-stage nitritation-anammox granular reactor.

2. Materials and methods

2.1. Reactor establishment and operation

A 2-L lab-scale sequential batch reactor (SBR) (height of 1000 mm and inner diameter of 58 mm) was operated continuously at 37 °C (Fig. 1S). The volumetric exchange ratio was 50% and the hydraulic retention time was fixed at 0.5 day. The reactor was configured at 6 h cycle: 5 min for feeding, 345 min for aeration, 5 min for settling and 5 min was allocated for effluent withdrawal. A programmable relay (ZEN-10C1AR-A-V2, Omron, Japan) was used to control the actuations of the air and water pumps, and regulated the different phases of the operational cycle. Air was supplied at a flow rate of 150 ml min^{-1} by a diaphragm pump (Laboport N86, KNF, Japan). A ceramic air diffuser located at the bottom of the reactor was used for formation of small bubbles. DO and pH of the reactor was about 1.0 mg L^{-1} and 7.6, respectively. Reactor was inoculated with nitrifying biomass (3 g-volatile suspended solid (VSS) L^{-1} taken from a PN reactor (Rathnayake et al., 2013) and anammox biomass (5 g-VSS L^{-1}) taken from an up flow column reactor (Tsushima et al., 2007). The reactor was fed with synthetic wastewater contained; NH_4^+ (about 300 mg-N L^{-1}), $CaCl_2$ 100 mg L^{-1}, $MgSO_4$ 300 mg L^{-1}, KH_2PO_4 30 mg L^{-1}, $KHCO_3$ 1500 mg L^{-1} and trace element solutions (van de Graaf et al., 1995). At steady state operation, an average granular size was 2.42 ± 1.48 mm (ca. 70% of granules have diameters between 1.5 and 3.5 mm).

2.2. Nitrous oxide measurement

Real-time N_2O concentrations in the off-gas from the reactor were measured with a photo acoustic field gas monitoring devise (1412, INNOVA, Copenhagen, Denmark). In addition, N_2O concentrations in the representative off-gas samples were measured using a gas chromatography equipped with electron capture detector (GC-2014, Shimadzu, Kyoto, Japan). The effluent water samples were collected and analyzed by N_2O headspace technique as described previously (Elkins, 1980; Okabe et al., 2011a).

2.3. Next generation sequencing

16S rRNA gene amplicon deep sequencing was conducted using the MiSeq technology (Illumina, Hayward, CA) to analysis microbial community structure. Genomic DNA was extracted from the nitritation-anammox granules (n = 3) using a PowerSoil DNA Isolation kit (MoBio Technologies, Carlsbad, CA). The partial 16S rRNA gene sequences including the V3 and V4 regions were then amplified using primers Bakt_341F and Bakt_805R (Herlemann et al., 2011) with Illumina overhang adaptor sequences attached to their 50 ends (Table S1) (Rathnayake et al., 2015). Twenty-five cycles of PCR were confirmed to be necessary and sufficient to reach the log-linear phase based on quantitative PCR analysis conducted using a KAPA SYBR Fast qPCR kit (Kapa Biosystems) and the Bakt_341F and Bakt_805R primers. The purified DNA was mixed with Phi X control DNA and employed as a template for paired-end sequencing using the MiSeq Reagent Kit v2 (500 cycles) and a MiSeq sequencer (Illumina). Sequence reads from triplicate samples were analyzed using QIIME 1.8.0 (Caporaso et al., 2010) with the Silva 119 database.