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Structure and composition of biofilm communities in a moving bed biofilm reactor for nitritation–anammox at low temperatures

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HIGHLIGHTS

- We studied a nitritation–anammox moving bed biofilm reactor at low temperatures.
- Shifts from 19 to 13 °C reduced nitrogen removal, at 10 °C removal was unstable.
- Anammox bacteria (at least two taxa of *Brocadia* sp.) dominated the biofilm biomass.
- Aerobic ammonia oxidizing bacteria were fewer, located at the biofilm-water interface.
- Nitrite oxidizing bacteria were low in abundance, but had some activity.

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ABSTRACT

It is a challenge to apply anaerobic ammonium oxidation (anammox) for nitrogen removal from wastewater at low temperatures. Maintenance of anammox- and aerobic ammonia oxidizing bacteria (AOB) and suppression of nitrite oxidizing bacteria (NOB) are key issues. In this work, a nitritation–anammox moving bed biofilm pilot reactor was operated at 19–10 °C for 300 d. Nitrogen removal was decreasing, but stable, at 19–13 °C. At 10 °C removal became unstable. Quantitative PCR, fluorescence in situ hybridization and gene sequencing showed that no major microbial community changes were observed with decreased temperature. Anammox bacteria dominated the biofilm ($0.9–1.2 \times 10^{14}$ 16S rRNA copies m^{-2}). Most anammox bacteria were similar to *Brocadia* sp. 40, but another smaller *Brocadia* population was present near the biofilm-water interface, where also the AOB community (*Nitrosomonas*) was concentrated in thin layers ($1.8–5.3 \times 10^{12}$ amoA copies m^{-2}). NOB (*Nitrobacter*, *Nitrospira*) were always present at low concentrations ($<1.3 \times 10^{11}$ 16S rRNA copies m^{-2}).

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1. Introduction

Nitrogen removal from wastewater is necessary to prevent eutrophication of receiving water bodies. At wastewater treatment plants (WWTPs), nitrogen removal is commonly performed by nitrification–denitrification. An alternative route for biological nitrogen removal is provided by anammox-based processes (Siegrist et al., 2008). By combining nitritation of half of the ammonium to nitrite by aerobic ammonia oxidizing bacteria (AOB) and oxidation of remaining ammonium with anammox bacteria, using the formed nitrite as electron acceptor, ammonium can be removed as gaseous nitrogen (N_2) (Kartal et al., 2010). One main benefit of anammox-based processes lay in the fact that organic carbon is not used for nitrogen removal. The organic carbon in wastewater constitutes a resource that instead can be used for e.g. energy production as biogas. Conventional nitrogen removal based on denitrification is dependent on organic carbon and in many cases extra organic carbon has to be added to the wastewater to provide denitrifying bacteria with carbon and energy. Another large benefit with anammox processes is the reduced need for aeration, since only half of the ammonium is oxidized by AOB with oxygen as electron acceptor. Aeration is responsible for a large part of the energy consumption of WWTPs. The combined effects of improved utilization of organic matter for production of biogas and reduced aeration can in fact transform WWTPs from consumers of energy into net producers of energy (Kartal et al., 2010). Other benefits with the anammox-based processes include decreased sludge production, which is a major cost at WWTPs, as

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well as decreased emission of the greenhouse gases CO_2 and N_2O (Kartal et al., 2010; Siegrist et al., 2008).

Anammox-based processes are today implemented at several WWTPs for treatment of reject water (dewatered sludge liquor) from anaerobic sludge digestion, where the high temperature (often around 30 °C) and the ample supply of ammonium (>500 mg N L^{-1}) provide beneficial conditions for the slow growing anammox bacteria (van der Star et al., 2007). Furthermore, the high temperature enables partial nitrification by washout of unwanted nitrite oxidizing bacteria (NOB), which have a lower growth rate than AOB at temperatures above 20–25 °C (Hellinga et al., 1998). However, in the main stream of wastewater, which contains 80–85% of all nitrogen at WWTPs, the more challenging conditions of lower temperatures, less substrate and presence of residual organic carbon has so far hindered implementation of anammox-based processes for nitrogen removal. Also for nitrogen removal of landfill leachates (Cema et al., 2007) and effluent from psychrophilic anaerobic digesters (Vazquez-Padin et al., 2009), the low temperatures provide a challenge for anammox-based processes. At the low temperatures, the slow growth rates and low activities of anammox bacteria and AOB decrease the removal capacity significantly (Isaka et al., 2008; Vazquez-Padin et al., 2011) and make biomass retention crucial (Vazquez-Padin et al., 2011). Furthermore, at low temperatures partial nitritation is harder to achieve since NOB are expected to have a higher growth rate than AOB at 10–20 °C (Hellinga et al., 1998). Despite these challenges, lab-scale reactors with anammox processes have been successfully operated at temperatures of 20 °C or lower (Cema et al., 2007; Dosta et al., 2008; Isaka et al., 2008; Vazquez-Padin et al., 2011). Growth of anammox bacteria resulting in establishment of an anammox process have been demonstrated at 20 °C (Hendrickx et al., 2012) and in a recent study, stable long-term operation of a nitritation–anammox reactor at 12 °C and low influent ammonium concentrations was achieved in a lab-scale SBR by careful process control (Hu et al., 2013). However knowledge is still lacking in order to operate stable full-scale reactors at low temperatures for e.g. treatment of the main stream of wastewater. In particular, little attention has been paid to the detailed response of the microbial community to temperature changes.

Here, a one-stage nitritation–anammox pilot MBBR (200 L) receiving reject water from anaerobic sludge digestion was operated at gradually decreasing temperatures from 19 to 10 °C. Long-term effects on the community composition of nitrogen converting microorganisms and their structure in the biofilms was investigated and discussed in relation to the function of the MBBR. Microbial analysis was performed using quantitative PCR (qPCR), fluorescence in situ hybridization (FISH) combined with confocal laser scanning microscopy (CLSM) and sequencing of 16S rRNA- and amoA gene clone libraries.

2. Methods

2.1. The pilot plant

The MBBR pilot plant was situated at the Centre for municipal wastewater purification (Hammarby Sjöstadsverk), Stockholm, Sweden and received reject water from anaerobic sludge digestion. The pilot MBBR had a volume of 200 L. It was filled to 40% with Kaldnes K1® biofilm carriers (specific surface area 500 m^2 m^{-3}). The biofilm carriers were kept in motion by stirring with mixers (50 rpm) resulting in a velocity gradient of 184–208 s^{-1}, calculated according to Tchobanoglous et al. (2003), assuming a power number (N_p) for the impellers of 3. Air supply, from the bottom of reactor, was controlled via the dissolved oxygen (DO) concentration by a PID controller. The shear rate in the reactor caused by aeration was calculated at 31–35 s^{-1} according to Gapes and Keller (2001). Temperature (10–19 °C) was controlled by a cooler and a heater connected to a thermostat. The pilot reactor was operated at nitrogen loading rates (NLR) of 1–2.9 g NH_4^+–N m^{-2} d^{-1}.

2.2. Chemical measurements

The pilot MBBR was equipped for on-line measurements (Cerlic Controls AB, Sweden) of conductivity, pH, redox and DO. Samples of influent and effluent were taken and filtered (0.45 μm) for analysis of inorganic nitrogen species and chemical oxygen demand (COD) using Dr. LANGE cuvettes on a XION 500 spectrophotometer (HACH LANGE).

2.3. DNA extraction

At each sampling occasion DNA was extracted from three carriers. From each carrier, 30 mg of biofilm was used for extraction using the FastDNA spin kit for soil (MP Biomedicals, France) according to the manufacturers' recommendations. The concentration of the extracted DNA was measured using a NanoDrop ND-1000 spectrophotometer (ds DNA; 50 ng μl^{-1} at 260 nm, Thermo Scientific).

2.4. Quantitative PCR

Quantitative PCR (qPCR) was carried on an iQ5 Bio-Rad thermal cycler using the SYBR green chemistry. Each 25 μl qPCR reaction contained 12.5 μl iQ SYBR green supermix (Bio-Rad Laboratories), 3 μM of forward and reverse primers respectively and 10 ng target DNA. For primers sequences, annealing temperatures and primer references, see Table S1. The qPCR protocols for all primer pairs were as follows: 95 °C for 3 min; 40 cycles of 95 °C for 15 s, annealing temp. for 30 s, 72 °C for 30 s and 80 °C for 30 s (data acquisition); 72 °C for 7 min; and a melting curve at 72–95 °C with 0.5 °C increments, each for 30 s. Decimal dilutions of linearized plasmid target gene inserts were used as standards. The qPCR reactions were verified by single melting peaks and by gel electrophoresis for bands of the expected size. To test for possible inhibition of the PCR reaction by the DNA extracts, separate sets of reactions were run where standard plasmid inserts of DNA were added to the DNA extracts before the qPCR together with control reactions containing standard plasmid DNA added to water. No difference in cycle threshold values was observed between the DNA extracts and the controls, showing that the qPCR reactions were not inhibited.

2.5. Phylogenetic analysis

For construction of clone libraries, pooled DNA extracts from two sampling occasions (Nov. 2011 and Aug. 2012) were used. The amoA gene of AOB was amplified using primers amo1F/2R and the 16S rRNA gene of anammox bacteria was amplified using primers Pla46F-1390R (Table S1). PCR products were purified using Qiaquick (Qiagen) prior generation of clone libraries using the TOPO TA cloning kit for sequencing (Invitrogen) as recommended by the manufacturer. From each sampling occasion, 47 and 95 clones were selected for sequencing of the amoA and 16S rRNA gene, respectively, after amplification of the vector inserts using standard M13F/R primers. Sequencing was performed at Macrogen Inc. The sequences were assembled and trimmed using DNA Baser v2.9 (Heracle BioSoft) prior to alignment using ClustalW in MEGA5 (Tamura et al., 2011). Chimeric sequences, as identified by Bellerophon (Huber et al., 2004), were excluded from subsequent analyses. The amoA genes were aligned as amino acid sequences with 100% dereplication, while the 16S rRNA genes were dereplicated

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