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Bacterial structure of aerobic granules is determined by aeration mode and nitrogen load in the reactor cycle

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HIGHLIGHTS

- Aeration mode and N load determined the bacterial structure of the aerobic granules.
- Supernatant was treated most efficiently in the reactor with the longest anoxia.
- Anoxic phases in the reactor cycle promoted heterotrophic nitrification.
- Bacteria in granules were more evenly distributed among taxa under constant aeration.
- *P. aminophilus* predominated with 1 anoxic phase/cycle, and *Corynebacterium* sp. with 2.

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ABSTRACT

This study investigated how the microbial composition of biomass and kinetics of nitrogen conversions in aerobic granular reactors treating high-ammonium supernatant depended on nitrogen load and the number of anoxic phases in the cycle.

Excellent ammonium removal and predomination of full nitrification was observed in the reactors operated at 1.1 kg TKN m^{-3} d^{-1} and with anoxic phases in the cycle. In all reactors, *Proteobacteria* and *Actinobacteria* predominated, comprising between 90.14% and 98.59% of OTUs. Extracellular polymeric substances-producing bacteria, such as *Rhodocyclales*, *Xanthomonadaceae*, *Sphingomonadales* and *Rhizobiales*, were identified in biomass from all reactors, though in different proportions. Under constant aeration, bacteria capable of autotrophic nitrification were found in granules, whereas under variable aeration heterotrophic nitrifiers such as *Pseudomonas* sp. and *Paracoccus* sp. were identified. Constant aeration promoted more even bacteria distribution among taxa; with 1 anoxic phase, *Paracoccus aminophilus* predominated (62.73% of OTUs); with 2 phases, *Corynebacterium* sp. predominated (65.10% of OTUs).

1. Introduction

The technology of aerobic granular sludge has attracted the attention of researchers in recent years as a promising alternative to treatment of wastewater with activated sludge, because aerobic granular sludge provides better nutrient removal and secondary clarification. Aerobic granular sludge has a compact spherical structure that is formed by the spontaneous immobilization of different microorganisms in an exopolymer matrix. The layered structure of the granules creates the oxic and anoxic zones necessary for simultaneous nitrification and denitrification in the reactor.

When using anaerobic granular sludge to purify hard-to-treat wastewater, such as wastewater with a low COD/N ratio and high ammonium concentration, treatment performance can be improved by adjusting the reactor cycle. Modifying the granular sludge batch reactor (GSBR) cycle by changing the aeration regime is an easy and cheap way of increasing the efficiency of nitrogen removal by aerobic granules. During the anoxic phases denitrification takes place throughout the entire granule structure, which supports better nitrogen removal. In general, DO (dissolved oxygen) in granular sludge reactors is important because low DO concentrations negatively affect the formation and stability of aerobic granules due to oxygen diffusion limitation (McSwain and Irvine, 2008). However, by introduction of a pre-anoxic phase in the GSBR cycle, Wan et al. (2009) obtained successful aerobic granulation despite a reduced aeration rate. When a 30-min anoxic phase with pulse-feeding was added to the cycle before the aerobic phase, nitrogen removal from pig manure increased from 20% to 60% (Val del Río et al., 2013). It can be concluded that if the length and number of anoxic phases are properly chosen, complete nitrification and efficient denitrification will take place and operating

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costs of wastewater treatment can be reduced by shortening the aeration period. Another important factor that must be taken into consideration when focusing on efficient nitrogen removal in GSBRs is nitrogen load, which determines treatment efficiency and the final products of nitrification.

The types of processes taking place in the reactor depend on the species structure of the communities inhabiting that particular wastewater treatment system. Structural shifts in the microbial community that are associated with changes in the operational conditions of the biological system may help identify members of that ecosystem that are important for efficient wastewater treatment. There are reports showing dependencies between the structure of bacterial communities and operational parameters, geographical location, and wastewater composition in both laboratory-scale reactors and full-scale plants (Ye et al., 2011; Wang et al., 2012).

Some reports have provided clues about the microbial bases of the successful operation of GSBRs with a high nitrogen load. When high-ammonium wastewater was efficiently nitrified and denitrified in a GSBR with alternating 2 h oxic and 2 h anoxic modes, both nitrifiers and heterotrophs were present in the outside parts of granules; in contrast, with continuous aeration mode the denitrification rate was inefficient because only nitrifiers were on the outside of the granule structure and the heterotrophs were inside, which limited the rate of mass transfer in the granule (Adav et al., 2009). It was observed that at high ammonium load, anoxic/oxic conditions in the GSBR cycle stimulated the simultaneous activity of ammonium oxidizing bacteria (AOB), N_2O-reducers, and Anammox bacteria in aerobic granules (Cydzik-Kwiatkowska and Wojnowska-Baryła, 2014). However, there is no detailed information on the major bacterial groups that create the granule structure and how their relative number changes in response to changes in the oxygen concentration during the batch cycle of the GSBR. In addition to the practical applications of this knowledge, determination of how changes in the aeration regime modify the microbial composition of granules and which groups of bacteria are sensitive or relatively resistant to changes in the aeration regime or nitrogen load may also have implications for studies of the ecology of complex microbial biofilms.

Thus, the aim of this study was to examine how the structure of bacterial consortia and the kinetics of nitrogen removal in aerobic granular sludge reactors change with increases in the number of anoxic phases in the cycle and changes in nitrogen load. For molecular analysis of aerobic granules, pyrosequencing developed by Roche 454 Life Sciences (USA) was used. The presented results help to shed light on both the microbial structure of biomass in reactors with aerobic granules and on ecological questions such as which groups of bacteria predominate in these multi-species communities depending on the aeration mode and nitrogen loads.

2. Methods

2.1. Substrate

For substrate, digester supernatant was obtained from the municipal wastewater treatment plant in Olsztyn (Poland). The concentrations of pollutants were about 800 mg COD L^{-1} (ranging from 380 to 850 mg COD L^{-1}), 470 mg N-NH_4^+ L^{-1}, 570 mg N L^{-1}, 12 mg P L^{-1}. To ensure nitrification, carbonates and carbohydrates were added to the supernatant in theoretical amount. Since the COD in the supernatant was due to slowly-biodegradable organics, 800 mg COD L^{-1} of sodium acetate was added to the supernatant as an external carbon source for biomass synthesis and the electron donor for denitrification.

2.2. Experimental design

The experiment was conducted in four column GSBRs with a working volume of 4.5 L and a height to diameter ratio of 10. The reactors were operated by programmable logic controllers at a volumetric exchange rate of 63% $cycle^{-1}$ and a temperature of 26 °C. Air was supplied via aeration grids in the bottom of the GSBRs. Aeration phases were run with up-flow superficial air velocity of 0.8 cm s^{-1} and free dissolved oxygen evolution up to saturation. The reactors were seeded with aerobic granular sludge cultivated with the digester supernatant during a previous experiment. The sludge retention time was not controlled.

All GSBRs in the experiment had an 8 h cycle, for a hydraulic retention time (HRT) of 13 h. Filling, settling, and decantation of each lasted for 5 min. The first reactor was fed with a 50/50 mixture of supernatant and tap-water and underwent constant aeration. The second reactor also underwent constant aeration but was fed with (100%) undiluted supernatant. Both third and fourth GSBRs were fed with undiluted supernatant but the aeration times differed. The third reactor had one 45 min anoxic phase at the beginning of the cycle. The fourth reactor's cycle had two 45 min anoxic phases, one at the beginning and the second after 2.5 h. For simplicity, the reactors will be abbreviated with a subscript that refers to percentage of supernatant in the influent and the number of anoxic phases during the cycle; thus, first reactor will be abbreviated as R_{50_0}; second, R_{100_0}; third, R_{100_1}; and fourth, R_{100_2}.

Operational parameters in the experimental series are given in Table 1. During the reactor operation the influent and the effluent were analyzed. To determine the rates of changes in pollutant concentrations in each reactor, measurements were performed every hour throughout the cycle during the period of stable reactor performance. For efficiency calculations, the technological results obtained during the last 30 days of the stable GSBRs operation were taken. Nitrification efficiency was calculated as a percentage of oxidized nitrogen forms to TKN less the N used for biomass synthesis, while denitrification as a percentage of N reduced to all oxidized nitrogen forms in the reactor. The organic fraction of biomass (VSS) made up from 51% of TSS in R_{50_0} to 69% of TSS in R_{100_1}.

2.3. Analytical methods

Wastewater and biomass in the reactors were analyzed in accordance with APHA (1992). The oxygen concentration in the reactor was determined using a YSI ProODO™ probe (YSI). The settling properties of the granular sludge were determined using the sludge volumetric index after 5 min sedimentation (SVI_5).

2.4. Pyrosequencing

To investigate microbial structure in the aerobic granules depending on the operational conditions in the reactors, two biomass samples were taken from each GSBR during the period of its stable performance. DNA was isolated from both samples and then the isolates were mixed. DNA was extracted from approximately 300 mg of centrifuged granular sludge using a FastDNA® SPIN®Kit (Q-BIOgene). The concentration of the DNA was measured using a BioPhotometer (Eppendorf). The DNA isolated from biomass samples was of high purity and its concentrations were in the range of 180–210 μg mL^{-1}.

The V6 to V8 region of the bacterial 16S rDNA gene was amplified using the primer set 926f/1392r and sequenced using the Roche 454 FLX+ platform (Research and Testing Laboratory, Lubbock, USA). The raw data were placed in NCBI as BioProject ID SRP050531 (http://www.ncbi.nlm.nih.gov/). Chimera detection and removal from the raw pyrosequence reads was performed by