

Diversity and abundance of denitrifying bacteria in a simultaneously nitrifying and denitrifying rotating biological contactor treating real wastewater at low temperatures

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Abstract

Nitrification and denitrification processes occur simultaneously in aerobic wastewater biofilms. Although wide regions of the world have average temperatures of less than 15 °C for a half year, few studies have investigated the nitrogen removal by nitrification and denitrification in a single-stage aerobic biofilm reactor used for treating real wastewater under low-temperature conditions. This study showed successful wastewater treatment in a high average nitrogen removal rate of 78% at low water temperatures by simultaneous nitrification and denitrification in a rotating biological contactor (RBC) biofilm. Batch operations using the RBC to evaluate the rates of ammonium decrease at low temperatures demonstrated that the rate of ammonium decrease at 8 °C was 76% of that at 20 °C. Daily monitoring of nitrification and denitrification rates suggested that the denitrification rate was highly correlated with the nitrification rate. Next-generation sequencing (NGS) analysis revealed the presence of diverse and abundant denitrifying bacteria and aerobic bacteria in the RBC biofilm more than those in the activated sludge samples, which probably enabled the achievement of the high nitrogen removal rates at such low temperatures. Furthermore, correlation with the colony counts showed that the NGS analysis had the quantification range of three orders of magnitude (from 0.001% to 1%).

Key words: low temperatures, nitrogen removal, quantification of next-generation sequencing (NGS), real municipal wastewater, rotating biological contactor (RBC), simultaneous nitrification and denitrification (SND)

INTRODUCTION

Simultaneous nitrification and denitrification (SND) in a wastewater treatment process has been studied over the past four decades and has increasingly become a focus of attention in the last 10

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years because of the worldwide requirement for high water quality of discharged wastewater, at a smaller footprint and with less energy consumption. To achieve denitrification under aerobic conditions, it is important to carefully control the concentration of dissolved oxygen (DO) at a low level, especially in the case of suspended growth sludge such as in oxidation ditch systems (Metcalf & Eddy 2003). On the contrary, in the case of attached growth sludge such as biofilms, anaerobic zones are created inside the biofilm, even under submerged oxic or non-submerged conditions (Kühl & Jørgensen 1992; Okabe *et al.* 1999a). This is because oxygen in the surface layer of the biofilm is consumed by aerobic organisms, so that both nitrification and denitrification occur within the biofilm (Masuda *et al.* 1991; Satoh *et al.* 2003).

One of the biofilm-based wastewater treatment technologies is the rotating biological contactor (RBC). A partially submerged (typically 40%) RBC is effective in enhancing SND reaction because the RBC biofilm is periodically exposed to air and underwater conditions, so that nitrification and denitrification occur in turn, for instance, every 10 s, when the rotating speed is set at the optimum of 3 rpm (Watanabe *et al.* 1992). Thus, we believe that the RBC biofilm has great potential for achieving stably high performance of SND.

Most of SND studies have been conducted at temperatures of 25 °C, 30 °C, or higher. However, wide regions of the world (e.g., Europe, Central Asia, Eastern Asia, and Northern America) have average temperatures of less than 15 °C for a half year and less than 10 °C for a period of 3 months (Japan Meteorological Agency 2018). Only a few SND studies have been performed at such low temperatures (de Kreuk *et al.* 2005; Luostarinen *et al.* 2006). Moreover, there has been no study using RBC that focused on SND for nitrogen removal from real municipal wastewater at low temperatures. However, real wastewater at low temperatures should significantly affect the diversity of the key microbial groups of the RBC biofilm.

Nitrification and nitrifying bacteria have been studied in the RBC biofilm by cultivation-based techniques, 16S ribosomal RNA (rRNA)-based approaches and using microelectrodes. Since nitrifying bacteria are phylogenetically clustered in only several lineages of a 16S rRNA phylogenetic tree (Purkhold *et al.* 2003), they have been successfully identified by a fluorescent *in situ* hybridization (FISH) technique (Juretschko *et al.* 1998; Okabe *et al.* 1999b). In contrast, FISH and conventional cloning techniques have been impractical for the detection of denitrifying bacteria, which are phylogenetically spread over very diverse lineages (Graf *et al.* 2014). Therefore, information on the abundance and diversity of denitrifying bacteria involved in SND in the RBC biofilm is very limited. Currently, next-generation sequencing (NGS) allows analysis of a 100 or 1,000 times higher number of sequences than that analyzed by conventional cloning, which enables the comprehensive detection and analysis of relative abundance of denitrifying bacteria among total bacteria.

This study conducted a 2-month continuous operation of an aerobic RBC for real wastewater treatment and examined the performance of simultaneous nitrogen removal at low temperatures. Furthermore, to understand characteristics of the RBC biofilm, the diversity and relative abundance of denitrifying bacteria and nitrate-utilizing bacteria in the RBC biofilm were investigated based on NGS analysis of the microbial community and compared with those in several activated sludges from real wastewater treatment plants (WWTPs). Finally, the quantification range and resolution of the NGS analysis were assessed by comparing with the sequence read frequency with the *Bacillus* colony count.

MATERIALS AND METHODS

Rotating biological contactor

A bench-scale rotating-disk reactor consisted of five stainless steel mesh disks with a diameter of 0.6 m. The reactor volume was 73 L, and the hydraulic retention times (HRTs) were 8 h (until day

22) and 12 h (day 23–31), corresponding to the hydraulic loading rates of 3.23 and 2.15 L/m²/h, respectively. The disk rotational speed was 1.5 rpm. The disks were 40% submerged.

The reactor was operated for 2 months, from December 2016 to February 2017. To speed up the start-up process, the reactor was inoculated with 100 g of freeze-dried sludge from the excretion treatment plant (Minakami, Gunma, Japan), which was supplemented with 4 L of milk and minerals and then operated in a batch mode for 2 days. The influent was the toilet wastewater of the Gunma College of Technology. The average influent water quality is described in Table 1. The average influent biochemical oxygen demand (BOD) and NH₄⁺ concentration were approximately 300 and 40 mg/L, respectively.

Table 1 | Characteristics of influent water quality fed to the RBC reactor

Parameters	Average ± SD
BOD (mg/L)	298 ± 99
Suspended solids (mg/L)	102 ± 53
NH ₄ ⁺ (mg-N/L)	38 ± 11
NO ₃ ⁻ plus NO ₂ ⁻ (mg-N/L)	2.6 ± 2.1
PO ₄ ³⁻ (mg-P/L)	3.2 ± 1.4
Temperature (°C)	12.4 ± 2.8
DO	2.1 ± 0.4
pH	7.4 ± 0.2

Activated sludge samples

Activated sludge samples were collected from six domestic WWTPs and one pig farm WWTP: Gunma Kenou (GK) WWTP with a capacity of 240,000 m³/day, Nagaoka-Chuo (NC) WWTP with a capacity of 68,400 m³/day, Gunma Okutone (GO) WWTP with a capacity of 213,000 m³/day, Maebashi Omuro (MO) WWTP with a capacity of 756 m³/day, Maebashi Baba (MB) WWTP with a capacity of 200 m³/day, China Xining (CX) WWTP with a capacity of 85,000 m³/day, and Hayashi pig farm (HF) WWTP with a capacity of 180 m³/day and BOD of 5,500 mg/L. GK, NC, and GO operate the conventional activated sludge process. MO, MB, CX, and HF operate oxidation ditch, an intermittently aerated activated sludge, an aerated activated sludge after RBC, and an aerated lagoon, respectively.

Measurements of ammonium, nitrite, and nitrate

Ammonium was determined by the Nessler method after sample filtration to remove suspended particles. Similarly, nitrate plus nitrite were routinely determined after sample filtration by an ultraviolet spectrophotometric screening method (APHA, AWWA, WEF 2005). The concentration of each nitrate and nitrite was determined by a naphthyl-ethylenediamine spectrometric method combined with an automated cadmium reduction method (APHA, AWWA, WEF 2005) using an Auto Analyzer (BL Tec K.K., Tokyo, Japan) equipped with a cadmium reduction column. The data confirmed that nitrite was a minor component of ionic nitrogen, and its concentration was always below 1 mg-N/L.

The daily nitrification rate (mg-N/m²/h) was calculated as follows: (influent NH₄⁺-effluent NH₄⁺) × reactor volume/RBC surface area/HRT. The daily denitrification rate (mg-N/m²/h) was calculated

based on the following equation: $[(\text{influent NH}_4^+ - \text{effluent NH}_4^+) - (\text{effluent NO}_x^- - \text{influent NO}_x^-)] \times \text{reactor volume} / \text{RBC surface area} / \text{HRT}$.

Batch experiment for NH₄⁺ decrease rate and NO_x⁻ increase rate

The NH₄⁺ decrease rate and NO_x⁻ (NO₂⁻ plus NO₃⁻) increase rate were determined at 20 °C, 13 °C, and 8 °C by batch operation of RBC with the addition of an NH₄Cl solution to a final concentration of 20 mg-N/L at the beginning of the batch incubation. The concentrations of NH₄⁺ and NO_x⁻ during the initial 3-h batch incubation were used to calculate the NH₄⁺ decrease rate and the NO_x⁻ increase rate. The SND efficiency represents the disappeared NO₂⁻ plus NO₃⁻ (denitrification) per NO₂⁻ plus NO₃⁻ expected to be produced from the decreased NH₄⁺ (nitrification). The SND efficiency was calculated by subtracting the NO_x⁻ increase rate from the NH₄⁺ decrease rate and then dividing the value by the NH₄⁺ increase rate using the following equation:

$$\text{SND efficiency (\%)} = [1 - (\text{effluent NO}_3^- - \text{influent NO}_3^-) / (\text{influent NH}_4^+ - \text{effluent NH}_4^+)] \times 100$$

Enumeration of *Bacillus* population

One milliliter of an RBC biofilm sample was suspended in 20 mL of a sterile 0.9% (w/v) sodium chloride solution. For an activated sludge sample, 20 mL of a mixed liquor was used for the following procedure. The suspended biofilm sample or activated sludge sample was homogenized and serially diluted by a 10-fold dilution series with a sterile sodium chloride solution. One hundred microliters of a serially diluted sample were plated onto *Bacillus* agar medium (modified from Murakami *et al.* 1995), and the plates were incubated at 32 °C for 48 h. The *Bacillus* agar medium contained (per liter): nutrient broth, 8 g; glucose, 8 g; soluble starch, 10 g; sodium chloride, 6 g; agar, 17 g. The typical *Bacillus* colonies were counted as described by Hatamoto *et al.* (2017). Some of the colonies were picked and phylogenetically confirmed to belong to the genus *Bacillus* by 16S rRNA gene sequencing analysis.

16S rRNA gene sequencing

Samples collected from the RBC reactor and seven different activated sludge aeration tanks were used for microbial community analysis. The RBC samples were collected at day 0, day 4, and day 14 after a start-up period of 1 month when the biofilm thickness became constant. Each sample was analyzed as follows and averaged to calculate the relative abundance of denitrifying bacteria. One of the RBC samples at day 14 was sectioned into four layers using cryomicrotome (CM3050S, Leica Microsystems, Inc., Nussloch, Germany).

DNA extraction, polymerase chain reaction (PCR), NGS, and quantitative insights into microbial ecology (QIIME) analysis were carried out at the Bioengineering Lab. Co., Ltd (Atsugi, Japan). Genomic DNA was extracted using a FastDNA spin kit for soil (MP Biomedicals, Santa Ana, California, USA) according to the manufacturer's instructions. Then, the V3-V4 region of the bacterial 16S rRNA gene was PCR-amplified using the primers S-D-Bact-0341-b-S-17 and S-D-Bact-0785-a-A-21 (Herlemann *et al.* 2011) according to the Illumina, Inc., Part #15044223 Rev. B. The PCR products were sequenced using an Illumina MiSeq instrument. The sequence data were analyzed using QIIME (Caporaso *et al.* 2010). Approximately 50,000 reads of 430-bp long were obtained for each sample. A chimera check and operational taxonomic units (OTUs) clustering were performed, and then OTU representatives were analyzed phylogenetically against the Greengenes database (DeSantis *et al.* 2006).

RESULTS AND DISCUSSION

Performance of a continuous RBC reactor treating real wastewater at low temperatures

The water quality of the influent and effluent of the continuous RBC reactor was monitored for 2 months at water temperatures ranging from 6 to 18 °C during the winter period (Figure 1). The average water quality of the influent wastewater is summarized in Table 1. After a start-up period of 1 month, approximately 20 mg-N/L ammonium decreased by nitrification, whereas there was no equivalent increase of nitrate and nitrite, and thus 36% of total inorganic nitrogen was lost. The sum of the influent NH_4^+ and NO_x^- was 44 ± 10 mg-N/L, and the sum of the effluent NH_4^+ and NO_x^- was 28 ± 14 mg-N/L. The results indicated that part of the nitrate produced by nitrification was simultaneously denitrified in the RBC biofilm by a so-called SND process. The efficiency of SND was $69 \pm 15\%$ when operated at HRT of 8 h (until day 22) and $78 \pm 10\%$ when operated at HRT of 12 h (day 23–31) (Figure 1(c)). In the case of the conventional activated sludge process for aerobic wastewater treatment, the decrease in ammonium was simultaneous with the increase in nitrate by nitrification.

Figure 2 shows the correlation between daily nitrification and denitrification rates during the RBC operation. Although both rates fluctuated during the 30-day RBC operation, interestingly, the daily change of the denitrification rate completely coincided with the daily change of the nitrification rate (Figure 2(a)). In addition, the denitrification rates never exceeded the nitrification rates, even when a higher concentration of nitrate than that of ammonium was expected to exist in the bulk water, for example, after the peaks of the daily nitrification rate. The daily nitrification rates were highly correlated with the daily denitrification rates (Figure 2(b)), and the daily denitrification rates were at approximately 80% of the daily nitrification rates, based on the approximate line. These results demonstrated that the denitrification reaction was dependent on the nitrification reaction in the RBC biofilm. In other words, nitrification was a rate-limiting step in the SND.

Ammonium decrease rate and nitrate/nitrite increase rate at low temperatures

The ammonium decrease rate and nitrate/nitrite production rate were determined at low temperatures in a batch experiment using ammonium as a sole nitrogen compound. The ammonium decrease rates (i.e., nitrification rates) were 54, 56, and 44 mg-N/m²/h at temperatures of 20 °C, 13 °C, and 8 °C, respectively (Figure 3). Thus, the ammonium decrease rate at 8 °C was still 76% of that at 20 °C, suggesting that the nitrifying bacteria in the RBC biofilm were tenacious at such low temperatures. The difference between the NH_4^+ decrease rate and the NO_x^- increase rate reflects the denitrification rate, which is expressed as the SND efficiency in Figure 3. Although the SND efficiency was lower at lower temperatures, that at 8 °C was still 29%, i.e., approximately one-third of oxidized ammonium was denitrified in the aerobic RBC biofilm.

Phylogenetic diversity of denitrifying and nitrate-utilizing bacteria

Microbial communities of RBC biofilms were analyzed by NGS. The relative abundances of denitrifying bacteria, including nitrate-utilizing bacteria, in the microbial communities are shown in Figure 4(a) and Table 2. Many of the 16S rRNA gene sequences in the RBC biofilm were associated with denitrifying and nitrate-utilizing bacteria. The most abundant phylotypes were related to the genera *Rhodobacter* (relative abundance: 3.9%), *Zoogloea* (relative abundance: 1.9%), and *Leucobacter* (relative abundance: 1.9%), whose members are capable of denitrification (Weon *et al.* 2012; Bellini *et al.* 2013; Graf *et al.* 2014; Unz 2015). *Acidovorax*, *Hydrogenophaga*, and *Comamonas* (sum of relative abundances: 2.8%), which belong to Comamonadaceae, are well-known abundant phylotypes in activated sludge, many species capable of heterotrophic denitrification (Graf *et al.*

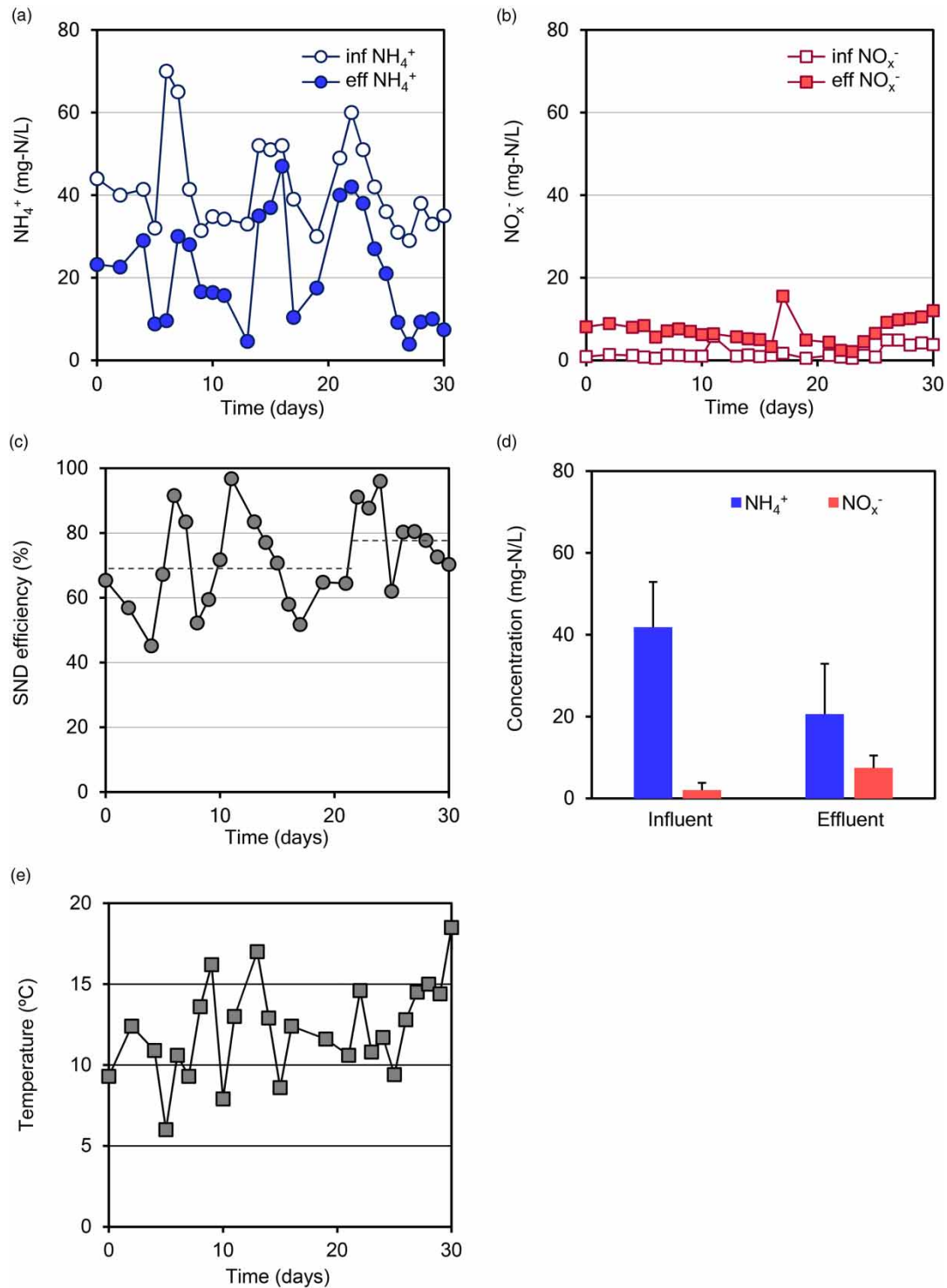


Figure 1 | Performance of the RBC reactor during the treatment of real wastewater at low temperatures after a start-up period of 1 month. (a) Time-course monitoring of influent and effluent ammonium concentrations. (b) Time-course monitoring of influent and effluent nitrite plus nitrate (NO_x^-) concentrations. (c) Time-course changes of the SND efficiency. Dot lines indicate average values obtained during periods of operating at HRT of 8 h (until day 22) and HRT of 12 h (from day 23 to day 30), respectively. (d) Average influent and effluent concentrations of NH_4^+ and NO_x^- . (e) Time-course monitoring of the influent water temperature.

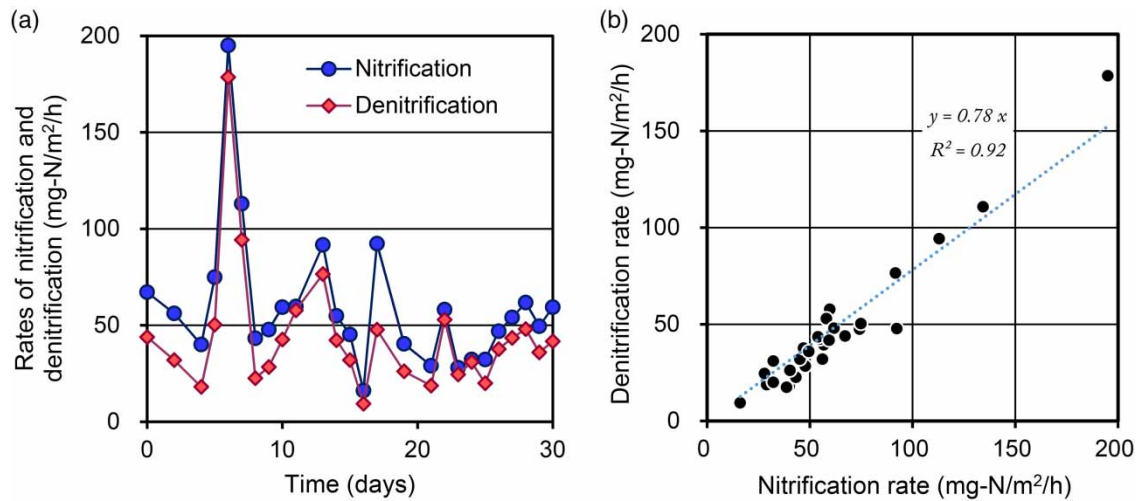


Figure 2 | Daily nitrification and denitrification rates of the RBC reactor after a start-up period of 1 month. (a) Time-course changes in daily rates of nitrification and denitrification. (b) Correlation between daily nitrification and denitrification rates. The dotted line is an approximate line described by the equation: $y = 0.78x$.

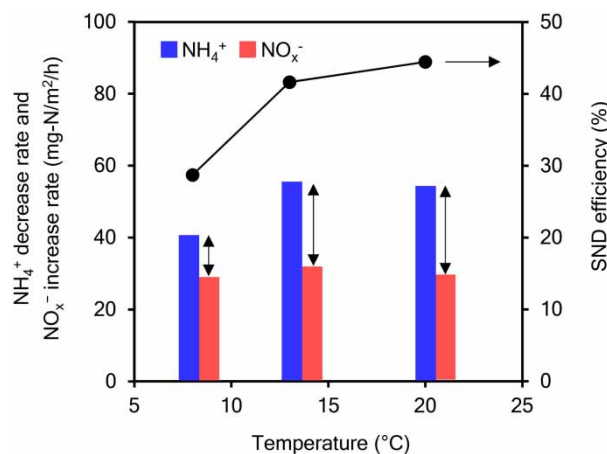


Figure 3 | Ammonium decrease rate, NO_x⁻ increase rate, and SND efficiency at 8 °C, 13 °C, and 20 °C, determined in a batch experiment using ammonium as a sole nitrogen compound. The arrows indicate the nitrogen loss by SND. Blue bars, ammonium decrease rate; red bars, NO_x⁻ increase rate; polygonal line, SND efficiency. NO_x⁻ is nitrite plus nitrate.

2014; Willems & Gillis 2015; Wu *et al.* 2015). Half of *Flavobacterium* species are able to reduce nitrate to nitrite (Bernardet & Bowman 2015). Denitrification ability of *Haliangium* (relative abundance: 1.3%) has been demonstrated by the microautoradiography–FISH technique (McIlroy *et al.* 2016). *Haliscomenobacter* (relative abundance: 1.3%), a thin-sheathed bacterium, has been shown to utilize ammonium and nitrate as nitrogen sources (van Veen *et al.* 1973). The genus *Bacillus* was one of the most abundant phylotypes. The BLAST analysis revealed that the most closely related sequences were those of *Bacillus subtilis* and *Bacillus tequilensis*, with a 100% match (both species have identical sequences in the sequenced region). Although *Bacillus* species are not well-known denitrifying bacteria, *B. tequilensis* has been shown to reduce nitrate to nitrogen gas (Gatson *et al.* 2006). *B. subtilis* reduces nitrate to nitrite and ammonium during nitrate respiration (Logan & Vos 2015). Some strains of *B. subtilis* have been reported to perform heterotrophic nitrification and denitrification (Kim *et al.* 2005; Yang *et al.* 2011). Thus, the RBC biofilm harbored a higher diversity and abundance of denitrifying bacteria and nitrate-utilizing bacteria.

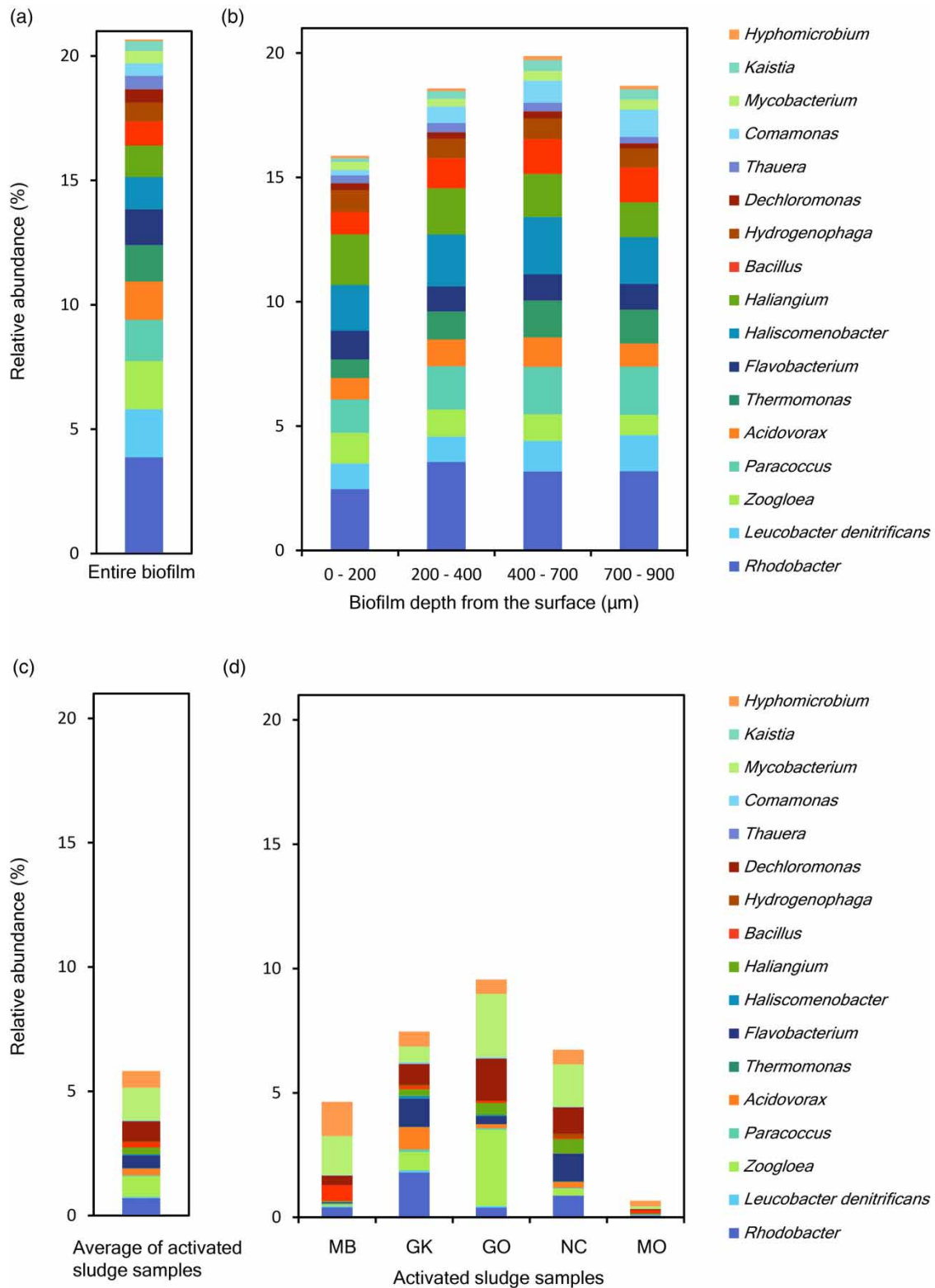


Figure 4 | Relative abundances of denitrifying bacteria in the RBC biofilm and in activated sludge samples. (a) Entire biofilm; (b) each layer of the biofilm, every 200 or 300 μm from the surface to bottom; (c) average relative abundance in five activated sludge samples; (d) activated sludge samples collected at five different locations.

Table 2 | Relative abundances of denitrifying bacteria and nitrate-utilizing bacteria in the RBC biofilm and activated sludge samples analyzed by NGS

Phylogenetic affiliation (class; genus)	Relative abundance (%) ^a		Ratio Biofilm/AS
	RBC biofilm ^b	Activated sludge ^c	
<i>Alphaproteobacteria; Rhodobacter</i>	3.9 ± 0.9	0.70 ± 0.67	6
<i>Betaproteobacteria; Zoogloea</i>	1.9 ± 0.6	0.83 ± 1.29	2
<i>Actinobacteria; Leucobacter</i> ^d	1.9 ± 0.7	0.04 ± 0.03	45
<i>Alphaproteobacteria; Paracoccus</i>	1.7 ± 0.5	0.05 ± 0.04	36
<i>Betaproteobacteria; Acidovorax</i>	1.5 ± 0.4	0.26 ± 0.36	6
<i>Gammaproteobacteria; Thermomonas</i>	1.5 ± 0.4	0.02 ± 0.01	83
<i>Flavobacteriia; Flavobacterium</i> ^e	1.4 ± 0.8	0.52 ± 0.56	3
<i>Saprosirae; Haliscomenobacter</i> ^f	1.3 ± 0.4	0.03 ± 0.05	39
<i>Deltaproteobacteria; Haliangium</i>	1.3 ± 0.8	0.28 ± 0.25	5
<i>Bacilli; Bacillus</i> ^g	1.0 ± 0.8	0.20 ± 0.25	5
<i>Betaproteobacteria; Hydrogenophaga</i>	0.8 ± 0.1	0.05 ± 0.07	15
<i>Betaproteobacteria; Dechloromonas</i>	0.5 ± 0.4	0.80 ± 0.63	0.7
<i>Betaproteobacteria; Thauera</i>	0.5 ± 0.3	0.01 ± 0.004	52
<i>Betaproteobacteria; Comamonas</i>	0.5 ± 0.3	0.03 ± 0.02	19
<i>Actinobacteria; Mycobacterium</i> ^e	0.5 ± 0.2	1.31 ± 0.95	0.4
<i>Alphaproteobacteria; Kaistia</i>	0.4 ± 0.1	0.003 ± 0.003	131
<i>Alphaproteobacteria; Hyphomicrobium</i>	0.1 ± 0.1	0.67 ± 0.42	0.1

^aAverage relative abundance ± standard deviation.^bAverage data for three mature biofilms from the RBC reactor collected on different days.^cAverage data for activated sludge samples collected from five locations.^dSequences affiliated with *Leucobacter denitrificans* (98% of 412 bp) were considered for this calculation.^eNO₃⁻ to NO₂⁻.^fNO₃⁻ assimilation.^gDenitrification ability is different among species.

Phylotypes related to denitrifying and nitrate-utilizing bacteria were significantly less abundant in the activated sludge samples. The relative abundance of total denitrifiers and nitrate utilizers in the RBC biofilm was 21%, whereas that in the activated sludge samples was 6% (Figure 4(a) and 4(c)). Most of the phylotypes affiliated with denitrifiers (14 of 17) were more abundant in the RBC biofilm than in the activated sludge samples (Table 2). The phylotypes affiliated with the genera *Leucobacter*, *Paracoccus* (van Spanning *et al.* 2015), *Thermomonas* (McIlroy *et al.* 2016), *Haliscomenobacter*, *Hydrogenophaga*, *Thauera* (Liu *et al.* 2013; Heider & Fuchs 2015), *Comamonas*, and *Kaistia* (Jin *et al.* 2012) were found in the RBC biofilm at an abundance of one or two orders of magnitude higher than that in the activated sludge samples. The relative abundances of the phylotypes affiliated with the genera *Rhodobacter*, *Acidovorax*, *Haliangium*, and *Bacillus* were five or six times higher in the RBC biofilm than in the activated sludge samples. These results confirmed that the RBC biofilm provided a more favorable habitat for denitrifying bacteria and nitrate-utilizing bacteria than did the activated sludge.

The depth profile of the denitrifying and nitrate-utilizing bacteria revealed that these bacteria were distributed throughout the biofilm, from the surface to the bottom, and were more abundant at a depth of 400–700 μm (Figure 4(b)). It seemed that the RBC biofilm was less suitable as a habitat for aerobic bacteria than was the activated sludge. However, the populations of *Bacillus*, *Flavobacterium*, and *Thermomonas*, which are generally recognized as aerobic bacteria, were also distributed throughout the depth of the biofilm. Microorganisms inhabiting the RBC biofilm are exposed to a periodic, ‘second-order’ change between oxic and anoxic conditions (Watanabe *et al.* 1992), in contrast to the bacteria living in the activated sludge, where the concentration of DO is relatively stable. The

time length of the alternating oxic and anoxic conditions is 20 s when the rotation speed is set at 1.5 rpm. The periodically changing conditions could enhance the abundance and diversity of denitrifying bacteria, nitrate-utilizing bacteria, and aerobic bacteria throughout the RBC biofilm.

Quantification by NGS

Bacillus colonies grown on *Bacillus* agar medium have morphologically distinctive features, and thus the *Bacillus* population was quantified by a colony-counting method. Figure 5 illustrates a logarithmic correlation between the *Bacillus* colony counts and the sequence read frequency of *Bacillus* determined by NGS. Based on the generated approximate line, 1% relative abundance of *Bacillus*-affiliated sequences corresponded to 1×10^9 *Bacillus* colony-forming units (CFU)/g mixed liquor suspended solids (MLSS). The correlation was described by the equation: $y = 10^9 \cdot x^{1.00}$. The one order of magnitude difference by colony counting was almost the same as the one order of magnitude difference by the NGS detection frequency. Although NGS is a high-throughput technology, DNA extraction and PCR are still unavoidable when analyzing microbial communities, and the bias caused by these steps affects the quantification of the relative abundance of each microbial population. Nevertheless, our NGS analysis had the quantification range of three orders of magnitude, from 0.001% to 1%, with the quantification resolution similar to that obtained by colony counting.

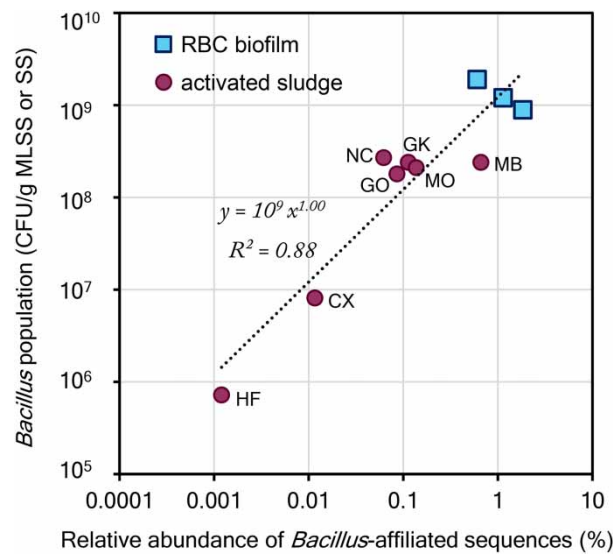


Figure 5 | Relative abundance of *Bacillus* species in the RBC biofilm. Correlation between the relative abundance of *Bacillus*-affiliated sequences and the cultivated *Bacillus* population in the RBC biofilm and seven activated sludge samples.

The *Bacillus* population of the RBC biofilm was, on average, $1.3 \pm 0.5 \times 10^9$ CFU/g suspended solids, while those of activated sludge were one or more orders of magnitude lower and were in the range from 7.2×10^5 to 2.7×10^8 CFU/g MLSS (Figure 5). Hatamoto *et al.* (2017) have recently reported that the *Bacillus* populations of 12 activated sludge samples were in the range between 1.0×10^7 and 6.6×10^8 CFU/g MLSS. Microbial community analysis using NGS also showed that the relative abundance of *Bacillus* species was the highest in the RBC biofilm ($1.2 \pm 0.6\%$) and was lower in the activated sludge samples (0.001–0.7%) (Figure 5). Thus, a higher abundance of *Bacillus* species in the RBC biofilm compared with that in the activated sludge samples was confirmed by both the colony-counting method and NGS.

Mechanism of SND in the RBC biofilm

Table 3 summarizes that the RBC reactor increased the efficiency of SND when operated in the continuous flow rather than in the batch mode and that high SND efficiency was obtained at lower ORP (oxidation–reduction potential) values. Although we did not investigate how ORP reached low values in the continuous RBC reactor, we expected that the ORP value was lowered by the high activity of both aerobic bacteria and denitrifying bacteria inhabiting the biofilm at all depths (Figure 4(b)). When influent BOD was continuously fed to the reactor, oxygen consumption by aerobic bacteria probably provided a favorable habitat for denitrifying bacteria in the RBC biofilm. On the other hand, in the batch mode operation, BOD was probably limited for these bacteria. Therefore, insufficient DO was consumed to drop the ORP value below zero, and so, SND in the batch mode operation did not increase to the levels of SND efficiency observed in the continuous flow operation. These observations suggested that the abundance of aerobic bacteria such as *Thermomonas*, *Flavobacterium*, and *Bacillus* species (Figure 4 and Table 2) was a key to achieve high SND efficiency in the RBC biofilm.

Table 3 | Summary of performance of the RBC reactor at low temperatures

Operation	Water temperature (°C)	SND efficiency (%)	NH ₄ ⁺ decrease rate ^b (mg-N/m ² /h)	NO _x ⁻ increase rate (mg-N/m ² /h)	DO (mg/L)	ORP ^a (mV)
Continuous flow ^b	12	73	68 ± 39	18 ± 9	1.9	-49
Continuous flow ^c	13	77	47 ± 13	11 ± 5	2.4	-11
Batch	8	29	41	29	2.7	113
Batch	13	42	56	33	5.2	84
Batch	20	44	54	30	6.5	71

SND, simultaneous nitrification and denitrification; DO, dissolved oxygen; ORP, oxidation–reduction potential.

^aHRT 12 h (until day 22).

^bHRT 8 h (from day 23 to day 30).

^cHRT 12 h (until day 22).

CONCLUSION

This study demonstrated highly efficient nitrogen removal by SND in a single-stage aerobic RBC reactor used for real wastewater treatment at low temperatures. The denitrification reaction was highly dependent on the nitrification reaction compared to the concentration of nitrate. NGS analysis revealed that the RBC biofilm harbored a higher diversity and an abundance of denitrifying bacteria, nitrate-utilizing bacteria, and aerobic bacteria than that by the activated sludge. Furthermore, these bacteria were distributed throughout the biofilm, from the surface to the bottom. The periodic, ‘second-order’ change between oxic and anoxic conditions in the RBC biofilm probably provided a favorable habitat for these bacteria and therefore enabled SND. The correlation between the colony counts and the sequence read frequency determined by NGS revealed that the NGS analysis had the quantification range of three orders of magnitude (from 0.001% to 1%).

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COMPETING INTERESTS

The authors declare no competing financial and non-financial conflicts of interest.

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