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Pyrosequencing analysis of microbial communities in hollow fiber-membrane biofilm reactors system for treating high-strength nitrogen wastewater

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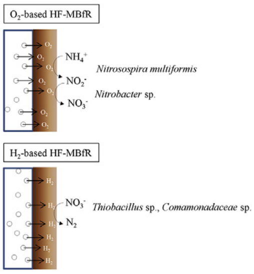
HIGHLIGHTS

- The nitrogen removal from wastewater was evaluated in autotrophic Hf-MBfR system.
- Only gaseous substrates such as O_2 and H_2 were supplied through a hollow fiber membrane.
- The pyrosequencing was performed for evaluation to establish autotrophic microbial community.
- The autotrophic microorganism was enriched within a relatively short period in Hf-MBfR.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewaters from swine farms, nitrogen-dealing industries or side-stream processes of a wastewater treatment plant (e.g., anaerobic digesters, sludge thickening processes, etc.) are characterized by low C/N ratios and not easily treatable. In this study, a hollow fiber-membrane biofilm reactors (HF-MBfR) system consisting of an O_2-based HF-MBfR and an H_2-based HF-MBfR was applied for treating high-strength wastewater. The reactors were continuously operated with low supply of O_2 and H_2 and without any supply of organic carbon for 250 d. Gradual increase of ammonium and nitrate concentration in the influent showed stable and high nitrogen removal efficiency, and the maximum ammonium and nitrate removal rates were 0.48 kg NH_4^+-N m^{-3} d^{-1} and 0.55 kg NO_3^--N m^{-3} d^{-1}, respectively. The analysis of the microbial communities using pyrosequencing analysis indicated that *Nitrosospira multiformis*, ammonium-oxidizing bacteria, and *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*, nitrite-oxidizing bacteria were highly enriched in the O_2-based HF-MBfR. In the H_2-based HF-MBfR, hydrogenotrophic denitrifying bacteria belonging to the family of *Thiobacillus* and *Comamonadaceae* were initially dominant, but were replaced to heterotrophic denitrifiers belonging to *Rhodocyclaceae* and *Rhodobacteraceae* utilizing by-products induced from autotrophic denitrifying bacteria. The pyrosequencing analysis of microbial communities indicates that the autotrophic HF-MBfRs system well developed autotrophic nitrifying and denitrifying bacteria within a relatively short period to accomplish almost complete nitrogen removal.

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1. Introduction

High-strength ammonia wastewater, e.g., effluents from sludge thickening and digestion processes, landfill leachate, and wastewater from fertilizer producers is not easily treatable by a conventional biological process, since it can exert potential ammonia toxicity and does not contain enough carbon to support complete denitrification (Pollice et al., 2002; Guo et al., 2009; Sun and Nemati, 2012). In general, a ratio of carbon: nitrogen is 2.7–3 for complete nitrate reduction. Low C/N ratio waste such as landfill leachate and fertilizers need additional carbon source for denitrification microbial process. Therefore, application of autotrophic denitrification is one of alternatives for high nitrogen content waste.

Recently, researches have been carried out to improve the O_2 transfer or utilization rate and to apply autotrophic denitrifiers to reduce NO_2^- and NO_3^- to N_2, for purifying nitrogen-rich wastewater (Sun et al., 2010). Recently, hollow fiber-membrane biofilm reactor (HF-MBfR) systems have been investigated to treat NH_4^+-rich wastewater (Lee and Rittmann, 2000, 2002; Martin and Nerenberg, 2012). The unique feature of this HF-MBfR system is that gas is supplied from the inside of membrane fibers to the liquid phase. Therefore, gas transfer flux to liquid phase can be better controlled to induce higher gas utilization, and achieve a higher nitrogen removal efficiency (Lee and Rittmann, 2000, 2002; Martin and Nerenberg, 2012). Absence of a liquid diffusion layer between the biofilm and the membrane induces higher gas transfer efficiency (Tang et al., 2010; Martin and Nerenberg, 2012). The gas is diffused via gas-permeable membrane from inner side of biofilm but ionic substrates such as NH_4^+ and NO_3^-, are supplied from the bulk liquid phase (counter-diffusion). Compared with a conventional biofilm system (co-diffusion), in the membrane-aerated biofilm reactor, the microbial composition of the biofilm is important for reactor performance (Lackner et al., 2010). Specially, the autotrophic nitrification and denitrification induce less biomass than heterotrophic treatment and improve nitrogen removal efficiencies by increasing substrate transport in the stratified biofilm (Beyenal and Lewandowski, 2000; Celmer et al., 2008). Previous studies reported energy savings as 40–75% comparing to conventional activated sludge (Suzuki et al., 1993; Semmens, 2005). However, coexistence of autotrophic and heterotrophic bacteria has been found in autotrophic nitrifying biofilms (Rittmann et al., 1994; Lebuhn et al., 2003). The microbial products by autotrophic nitrifying bacteria support the growth of heterotrophic nitrification.

An oxygen-based HF-MBfR (Eq. (1)) was used for the nitrification (Shin et al., 2005, 2008) and a hydrogen-based HF-MBfR (Eq. (2)), for removing oxidized nitrogen, e.g., nitrate through denitrification (Lee and Rittmann, 2000, 2003, 2002; Shin et al., 2005, 2008; Tang et al., 2010).

$$NH_4^+ + 1.863\ O_2 + 0.098\ CO_2 \rightarrow 0.0196\ C_5H_7NO_2 + 0.98\ NO_3^- + 0.941\ H_2O + 1.98\ H^+ \tag{1}$$

$$NO_3^- + H^+ + 2.86\ H_2 + 0.15\ CO_2 \rightarrow 0.0286\ C_5H_7NO_2 + 0.49\ N_2 + 3.14\ H_2O \tag{2}$$

According to Eqs. (1) and (2) (Hwang et al., 2010), stoichiometric oxygen and hydrogen requirements for nitrification and denitrification are 4.26 g O_2/g N and 0.41 g H_2/g N, respectively. In terms of alkalinity, autotrophic nitrification of 1 mg N L^{-1} NH_4^+ consumes 0.588 mg L^{-1} $NaHCO_3$ while 0.9 mg L^{-1} $NaHCO_3$ is required for autotrophic denitrification. Autotrophic hollow fiber-membrane biofilm reactors system could be optimized by preventing gas overconsumption without biofouling. Additionally, the study of microbial community structure and the identification of autotrophic bacteria in a real autotrophic HF-MBfR could help understanding the system performance in nitrogen removal. However, none of the papers has analyzed how the microbial community would develop in an autotrophic HF-MBfR.

Nitrogen is removed by two sequential microbial processes; the first process is nitrification in which NH_4^+ is oxidized (=nitrified) to NO_2^- and NO_3^- by autotrophs using O_2 as a terminal electron acceptor, and the second is denitrification in which NO_3^- or NO_2^- is reduced (=denitrified) to N_2 by heterotrophs using organic carbon as an electron donor. In previous studies on microbial communities in biofilms for nitrogen removal, ammonia-oxidizing bacteria (AOB) were dominant inside the biofilm (Hibiya et al., 2003) or only near the membrane (Cole et al., 2004). However, our previous work showed there was no local variation of microbial community in nitrification while a special variation in denitrification (Shin et al., 2015). In an aerobic fluidized bed reactor, *Nitrosomonas*-like bacteria was dominant in the granules after 300 d operation (Tsuneda et al., 2003). The analysis of microbial community in membrane-aerated biofilm reactors showed *Nitrosomonas* sp. was dominant (Gong et al., 2008). In a study where the impact of COD/N ratio on the microbial community structure of a membrane aerated biofilm reactor was investigated, only *Nitrosospira* could be detected at COD/N ratio of 5, while they were co-existed with *Nitrosomonas* at COD/N of 0 (Cydzik-Kwiatkowska and Wojnowska-Baryła, 2011). The structure of the ammonia oxidizer community did not change even at increased ammonium concentration (Mendum et al., 1999; Avrahami et al., 2002). *Nitrosomonas oligotropha*, which was the most abundant in the inoculated sludge, was often found in a biofilter (Wahman et al., 2011) or in a full-scale activated sludge process for sewage treatment (Dionisi et al., 2002; Limpiyakorn et al., 2006). This species well adapts to a low DO environment (Park and Noguera, 2004) and to a high N load condition (Cabrol et al., 2016). In a study on the stratification of AOB in a biofilm system, *Nitrosomonas* was found more abundant in the oxic-zone whereas *Nitrosospira* was found in the interface between the oxic and the anoxic zones (Schramm et al., 2000). *Nitrosospira multiformis*, autotrophic AOB relatively well adapt to an environment of high ammonium concentration (Jordan et al., 2005). For the analysis of nitrite-oxidizing bacteria (NOB), *Nitrobacter* outcompeted *Nitrospira* among NOB in a membrane-aerated biofilm reactor (Terada et al., 2010). *Nitrobacter* showed a higher specific activity than *Nitrospira* and it was dominant at a relatively high nitrite concentration (Kim and Kim, 2006; Haseborg et al., 2010) or in biofilm regions of 2 mg L^{-1} DO or greater (Downing and Nerenberg, 2008).

Denitrification biofilm showed a more complex microbial community structure. Autotrophic denitrifying bacteria (*Thiobacillus denitrificans*) constituted only 32% of all clones (Koenig et al., 2005) because microbial decay products could support the growth of heterotrophic denitrifying bacteria (Martin and Nerenberg, 2012). In an H_2-based MBfR for nitrate and perchlorate reduction, *Marinobacter hydrocarbonoclasticus* represented 53% of all clones in the MBfR biofilm (Van Ginkel et al., 2010). In a glass bead biofilm reactor using H_2 as an electron donor for denitrification, *Hydrogenophaga* sp. was a dominant species (Park et al., 2005). Previous studies show that the co-existence of autotrophs and heterotrophs in a hydrogenotrophic denitrification system is unavoidable, but it results in a higher denitrification efficiency (Lee et al., 2008; Zhao et al., 2012).

The pyrosequencing is a high-throughput DNA sequencing method to investigate the microbial community in a complex system such as wastewater treatment plants (Wang et al., 2012), activate sludge (Yu and Zhang, 2012), and river system (Xia et al., 2009). It obtains short segments of sequences via luminescent detection during the nucleotide incorporation (Ahmadian et al., 2006). In this study, we applied an autotrophic hybrid HF-MBfR system continuously fed with synthetic wastewater without organic carbon source for nitrogen removal; an O_2-based HF-MBfR was applied for nitrification, while an H_2-based HF-MBfR for denitrification. Biomass samples were collected at the point of each change and the pyrosequencing was performed with them to evaluate the development of autotrophic microbial community in each O_2 and H_2-based MBfR.

2. Materials and methods

2.1. HF-MBfR

Two identical reactors as HF-MBfR system were used: one for nitrification (the aerobic reactor) and the other for denitrification (the anaerobic reactor). Each reactor was made of clear acrylic plastic, and its height and diameter were 500 and 50 mm, respectively. One sampling port was made in the bottom. Membrane bundles (poly acrylonitrile) with a molecular weight cut-off of 300 kD were placed inside the rectors. High purity O_2 and H_2 were supplied via the membrane bundles for the nitrification and denitrification reactors, respectively, from the corresponding pressurized cylinder. The gas pressure was controlled at 30 kPa by a pressure regulator. The hollow fiber membrane had an outer diameter of 1.4 mm and an inner diameter of 0.8 mm. Each bundle was consisted of about 400 fibers, and the total outer surface area was 7037 cm^2. The total and working volumes of each reactor was 1 and 0.6 L, respectively. The reactor contents were continuously recirculated at the rate of 10 mL min^{-1} using a pump (MasterflexC/L, Vernon Hills, IL, USA). The temperature of the reactor was maintained by a heating tape (Daihan, Seoul, Korea) at 30 °C and the hydraulic retention time was maintained at 10 h during the operating period of 250 d. Activated sludge (mixed liquor suspended solid concentration: 2500 mg L^{-1}) from an aeration tank of a local domestic wastewater treatment plant was inoculated into each reactor at a level of 20% of the working volume. The reactor was fed with synthetic wastewater, composition of which is provided in Table S1 (Shin et al., 2005) and the pH of the feed medium was maintained at 7.0 ± 0.2. The synthetic wastewater was prepared by diluting the medium stock solution with tap water (residual chlorine: 0.1–0.3 mg L^{-1} as Cl_2). For a start operation, the ammonium sulfate (50 mg N L^{-1}) and sodium nitrate (30 mg N L^{-1}) were added to the medium as substrates for nitrification and denitrification, respectively. The influent nitrogen was gradually increased to prevent the inhibition of high concentration nitrogen to microbial metabolism. The reactor pH was monitored continuously with a pH meter. The inorganic carbon was supplied in the form of sodium bicarbonate to maintain inorg C/N ratio = 12 (Chiu et al., 2007) according to the influent nitrogen concentration.

2.1.1. O_2-based Hf-MBfR

Fig. 1a shows the performance of the O_2-based HF-MBfR. The NH_4^+ concentration of the influent to the reactor was initially set at 50 mg N L^{-1} and it was gradually increased up to 120 mg N L^{-1} until Day 50. NH_4^+ removal efficiency was also accordingly enhanced; it was maintained above 95% (Fig. 1a). The initial low NH_4^+ removal was attributed to a low amount of nitrifying bacteria. The pH dropped below 6 on Day 50 (See supporting information, Fig. S1) due to nitrification activity. For pH buffering, a phosphate buffering agent of 100 mM was added to the feed. The addition of the buffering agent increased the pH, but the NH_4^+ removal efficiency decreased down to 41% (Fig. 1a). The high concentration of phosphate buffering agent negatively affected the NH_4^+ removal efficiency. The addition of phosphate buffer was stopped and at the same time the influent NH_4^+ concentration was increased from 120 to 140 mg N L^{-1}, which again resulted in the pH drop to <6 (Fig. S1). We collected the biomass on Day 69 to investigate a possible change in the microbial structure after the phosphate shock (AeroNB1). When the removal efficiency recovered back to about 100% on Day 94, a biomass sample was also collected (AeroNB2). After Day 100, the diluted phosphate buffer (20 mM) was added into the synthetic wastewater, the pH of which was kept at 6.5–6.8 (Fig. S1). Even though the influent NH_4^+ concentration was gradually increased up to 200 mg N L^{-1} from Day 170 to Day 180, the removal efficiency was not affected (Fig. 1a). The maximum NH_4^+ removal rate was 0.48 kg NH_4^+-N m^{-3} d^{-1} according to Eq. (3), which is comparable with 0.30 kg NH_4^+-N m^{-3} d^{-1} obtained using a vertically moving biofilm system (Rodgers et al., 2006) and 1.5 kg NH_4^+-N m^{-3} d^{-1} using an aerobic upflow fluidized bed reactor (Tsuneda et al., 2003). The last microbial sample for the microbial community analysis was collected on Day 250 (AeroNB3), the last day of operation.

2.1.2. H_2-based Hf-MBfR

For denitrification, the initial concentration of nitrate in the H_2-base HF-MBfR was 30 mg N L^{-1}. One week after the start of the reactor operation, a dark blackish biofilm was formed on the membrane surface of the H_2-based HF-MBfR. Although the nitrate concentration in the feed was gradually increased up to 50 mg N L^{-1} over the first 60 d, in fact, the system continued to show a complete denitrification (Fig. 1b). We collected microbial sample at 69 d (AnaeroNB1). The nitrate removal rate was stabilized at 0.18 kg NO_3^--N m^{-3} d^{-1} on Day 91 and the nitrate loading rate was further increased by decreasing the HRT from 10 to 6 h, following relatively short duration of wastewater in anoxic tank (Yilmaz et al., 2008). The removal efficiency quickly decreased and the concentrations of nitrate and nitrite in the effluent increased to 0.075 and 0.065 kg N m^{-3}, respectively. On Day 92–94, the denitrification efficiency decreased down to 57% (Fig. 1b). When the HRT was returned back to 10 h, however, the denitrification efficiency of the reactor was recovered back to ~100% within 5 d (Fig. 1b).

Hydrogen was used as an electron donor for the reduction of NO_3^- to N_2 gas and the hydrogen supplementation via the hollow fiber-membrane might not be enough for the increased nitrate loading. For the verification, the impact of H_2 on denitrification efficiency was investigated by changing the amount of hydrogen supplied to the H_2-based HF-MBfR; the pressure of H_2 was reduced from 30 to 10 kPa on Day 115. Immediately after the H_2 pressure was lowered, nitrate started to be accumulated; the effluent NO_3^- concentration rose up to ~100 mg N L^{-1}. When the H_2 pressure was set back to 30 kPa, however, the denitrification efficiency was immediately recovered to the level before H_2 pressure had been lowered. Lastly, the influent nitrate concentration was raised up to 230 mg N L^{-1} from Day 130–170, and the NO_3^- removal efficiency was monitored. In short, the removal efficiency was not deteriorated; complete denitrification was continuously observed (Fig. 1b). The maximum denitrification rate was 0.55 kg NO_3^--N m^{-3} d^{-1}. For comparison (Ahmed et al., 2012), reported a denitrification rate of 0.33 kg NO_3^--N m^{-3} d^{-1} using an autotrophic biofilter, and (Park et al., 2002) achieved l. 3.9–4.5 kg NO_3^--N m^{-3} d^{-1} with their sulfur packed bed reactors. The microbial community analysis was collected on Day 250 (AneroNB2), the last day of operation, as that of O_2-base Hf-MBfR.

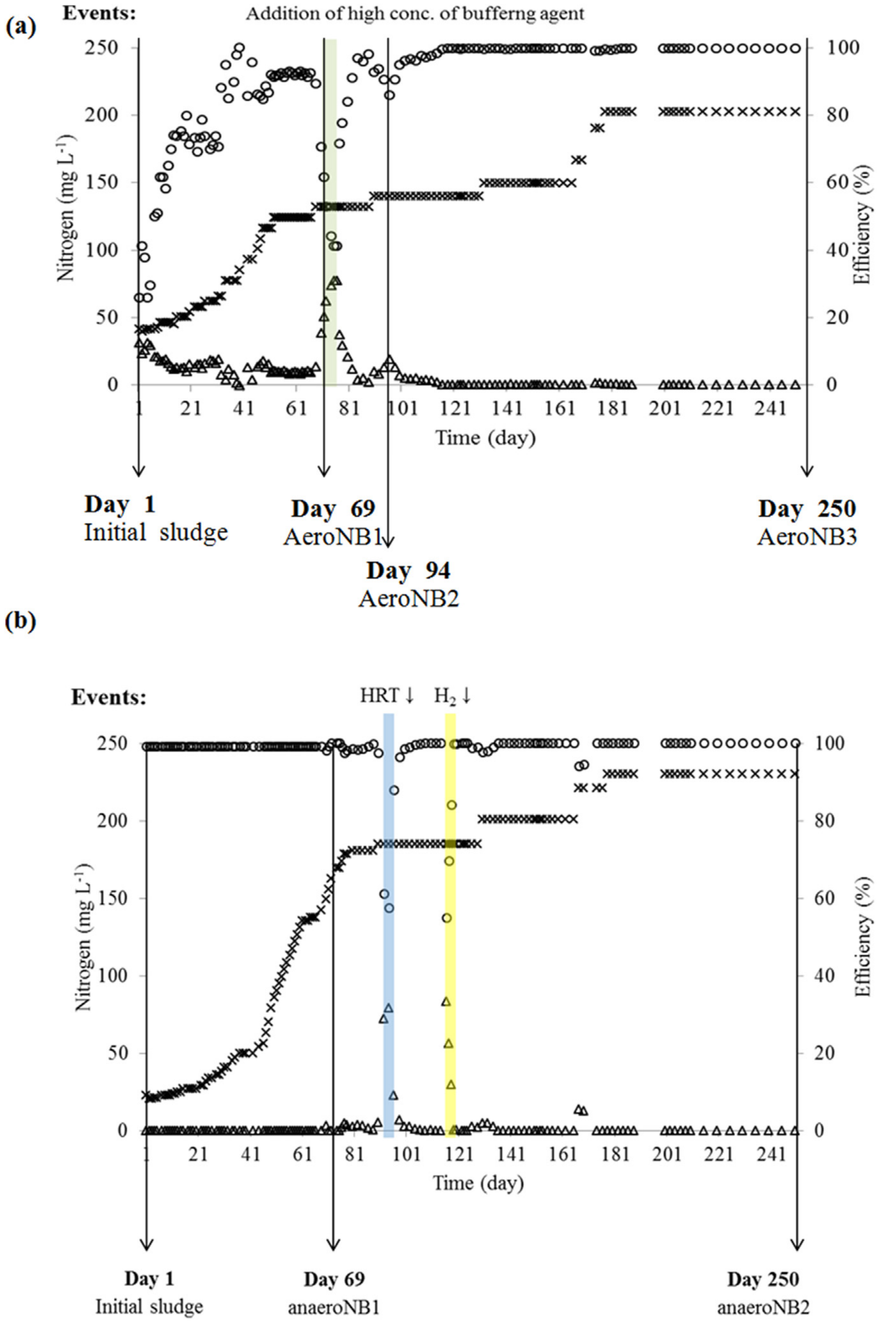

Fig. 1. The nitrogen removal in (a) the O_2-based HF-MBfR and (b) the H_2-based HF-MBfR. Ammonium-nitrogen removal shown as N-removal efficiency (○), NH_4^+-N or NO_3^--N concentration of influent (×) and effluent (△). The events are shown as vertical bars.

2.2. Nitrogen measurement

The concentrations of nitrate, nitrite, and ammonium were measured by colorimetry (Reflectoquant®, Merck, Darmstadt, Germany). A sample of 10 mL was collected on a daily basis to determine concentrations of the nitrogen species. The nitrogen removal rate (R) was calculated with the following equation.

$$R = \frac{Q([C]_i - [C]_e)}{V} \tag{3}$$

Where Q is flow rate ($m^3\ d^{-1}$), V is the volume of reactor (m^3), and [C] is NH_4^+ or NO_3^- concentration in the influent (i) or effluent (e).

2.3. Microbial community analysis using pyrosequencing

The enrichment of microbial communities for nitrification and denitrification was analyzed by pyrosequencing. Samples for community analysis were collected from the O_2-based HF-MBfR for nitrification on Days 1, 69, 94, and 250, and from the H_2-based HF-MBfR for denitrification on Days 1, 69, and 250. The total DNA was extracted with a Power Soil™ DNA isolation kit (Mo Bio Lab, Carlsbad, CA, USA), as described in the manufacturer's instruction. The 16S rRNA genes were amplified (Roche 454 GS FLX Titanium) using bar-coded universal primers for each sample. The universal bacterial primer was used for 16S rRNA gene amplification (27F: AGA GTT TGA TCM TGG CTC AG, 518r: WTT ACC GCG GCT GCT GG).

The amplification was carried out under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, with a final elongation at 72 °C for 5 min. The amplified products were purified with a QIAquick PCR purification kit (QIAgen, Valencia, CA, USA). The obtained readings from different samples were sorted by the unique barcode for each PCR product. The sequences of the barcode, linker, and primers were removed from the original sequencing reads. Potential chimera sequences were detected with the bellerophone method that compares the BLASTN search results of the forward half sequence and the reverse half. Reads were assigned against the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net) (Kim et al., 2012). The statistical analysis of sequence result including richness and diversity index was summarized in Table 1. Typical taxonomic suffixes are _s (for species), _g (genus), _f (family), _o (order), _c (class) and _p (phylum). “Unclassified taxons” were indicated with _uc. Interactive Krona HTML5 (Ondov et al., 2011) hierarchical and double pie chart community profiles have been included in the supplemental information (See SM1charts_supplemental.zip). Sequences identified in this study were deposited in the NCBI under BioProject PRJNA288193, BioSamples SAMN03788025, SAMN03788026, and SAMN03796064-SAMN03796067.

3. Results and discussion

In general, the microbial community structure developed in the HF-MBfR for nitrification consisted of 6 phyla: Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Nitrospirae, and Chloroflexi (Fig. S2). Nitrifying bacteria belonging to the proteobacterial phylum were most abundant in the nitrification reactor (Fig. S2). The microbial diversity decreased at the end of operation in O_2-based Hf-MBfR but increased in H_2-base Hf-MBfR as shown in Table 1. The rarefaction curves (Fig. S3) for samples, except from initial sludge, collected approach a plateau and it indicates samples could be as representatives of microbial species present in each condition.

3.1. *Nitrifying bacteria*

Fig. 2a shows the taxonomic composition of microbial community in the O_2-based HF-MBfR. The nitrifying bacteria (AOB + NOB) were computed as only ~ 0.4% of total microbial community in inoculated sludge (Fig. 2a, included in ETC; a collection of minor components of <3%). The autotrophic nitrifying bacteria increased and finally accounted for over 70% of the total bacterial community (Fig. 2a, Day 250). Especially, the AOB became the most abundant bacterial species. However, the NOB changed only 4% during the entire operation period after Day 69 (Fig. 2a). Fig. 2b showed the nitrifying bacterial composition in a species level in the O_2-based HF-MBfR. Interestingly, in the sludge initially used as the inoculum for the O_2-based HF-MBfR, NOB (colored bars) exceeded AOB (patterned bars) (Fig. 2b), which is against our common knowledge that AOB are more abundant than NOB. Probably, the environmental condition of the wastewater treatment system where the sludge was collected was more favorable for NOB's growth than AOB's (Schramm et al., 1999).

The major genus of AOB shifted from *Nitrosomonas* to *Nitrosospira* in the O_2-based HF-MBfR (Fig. 2b); the proportion of *Nitrosospira multiformis* gradually increased, and finally accounted for 99% of the total AOB (Fig. 2b). The enrichment with *Nitrosospira multiformis* already started at the initial phase (Day 69, Fig. 2b). With a relatively low diversity, ammonium oxidation mainly by *Nitrosospira multiformis* took place and showed a stable nitrogen removal even at the influent NH_4^+ concentration of >200 mg N L^{-1} (Fig. 1a).

After phosphate buffer was added to the feed, the biomass sample collected on Day 69 showed an increase of the abundance of nitrifying bacteria (Fig. 2a) and the major species of AOB and NOB were changed from *Nitrosomonas oligotropha* to *Nitrosospira multiformis*, from *Nitrospira defluvii* to *Nitrobacter winogradskyi* group, respectively (Fig. 2b). From the result, the environment of the O_2-based HF-MBfR was favorable for AOB, especially for *Nitrosospira multiformis*. *Nitrospira* was the major NOB in the inoculated sludge, but *Nitrobacter* was developed in the O_2-based HF-MBfR (Fig. 2b). However, the final biomass sample on Days 250 indicated that the dominant NOB species was *Nitrospira defluvii* as the same one shown in inoculated sludge (Fig. 2b). Therefore, it seems that *Nitrobacter*, such as *Nitrobacter winogradskyi* and *Nitrobacter vulgaris*, can thrive in a phosphate abundant condition. Nonetheless, the disturbance affected nitrification activity for only a short period, and the nitrification efficiency was almost recovered by Day 94 (AeroNB2). *Nitrospira* has a lower nitrite half-saturation constant (K_m), and it is more likely to dominate at a condition of a low ammonium or nitrite concentration compared to *Nitrobacter* (Blackburne et al., 2007). Therefore, *Nitrosospira multiformis* and *Nitrobacter* sp. (such as *Nitrobacter winogradskyi*, *Nitrobacter vularis*, *Nitrobacter hamburgensis*) could well adapt to the environment in the O_2-based HF-MBfR with the increased ammonium input. For a future work, the habitat stratification of nitrifying bacteria in the biofilm on the surface of hollow fiber membrane and the homogenous O_2-diffusion within biofilm should be investigated.

In the whole microbial community, *Rhodanobacter lindanoclasticus* was identified as the most abundant bacteria on Day 69 (Table 2, also see a hierarchical data browser Krona provided as Supplementary Information). The γ-Proteobacteria including *Rhodanobacter* sp. and *Xanthomonas* sp. utilized acetic acid and amino acids in autotrophic nitrifying biofilm and they were supported by nitrifiers (AOB and NOB) via the production of soluble microbial products (Kindaichi et al., 2004; Okabe et al., 2005). The temporal increase of *Rhodanobacter lindanoclasticus* seems to be related to the phosphate input, but it has not been reported elsewhere. *Xanthomonadaceae Rudaea* (Table 2) was involved in the hydrolysis of cellulose and urea (Weon et al., 2009) and able to utilize L-

Table 1
Summary of pyrosequencing data and statistical analysis of bacterial communities. Initial sludge is the inoculum (Day 1). AeroNB1, 2, and 3 are microbial samples collected on Days 69, 94, and 250, respectively from the nitrification reactor. AnaeroNB1 and NB2 are collected on Days 69, and 250, respectively from the denitrification.

	Initial sludge	Day 69	Day 94	Day 250	Day 69	Day 250
Number of reads	10,500	10,442	21,610	18,210	13,118	11,000
Average read length (bp)	446.8	443.3	446.8	459.0	444.7	442.8
Number of all contigs	1941	999	2202	2004	2158	1859
Chao1 richness	2689	1460	1010	905	1150	1237
Shannon index	6.12	4.86	4.68	3.61	4.60	4.77
Good's library coverage (1 = 100%)	0.93	0.96	0.99	0.99	0.98	0.97

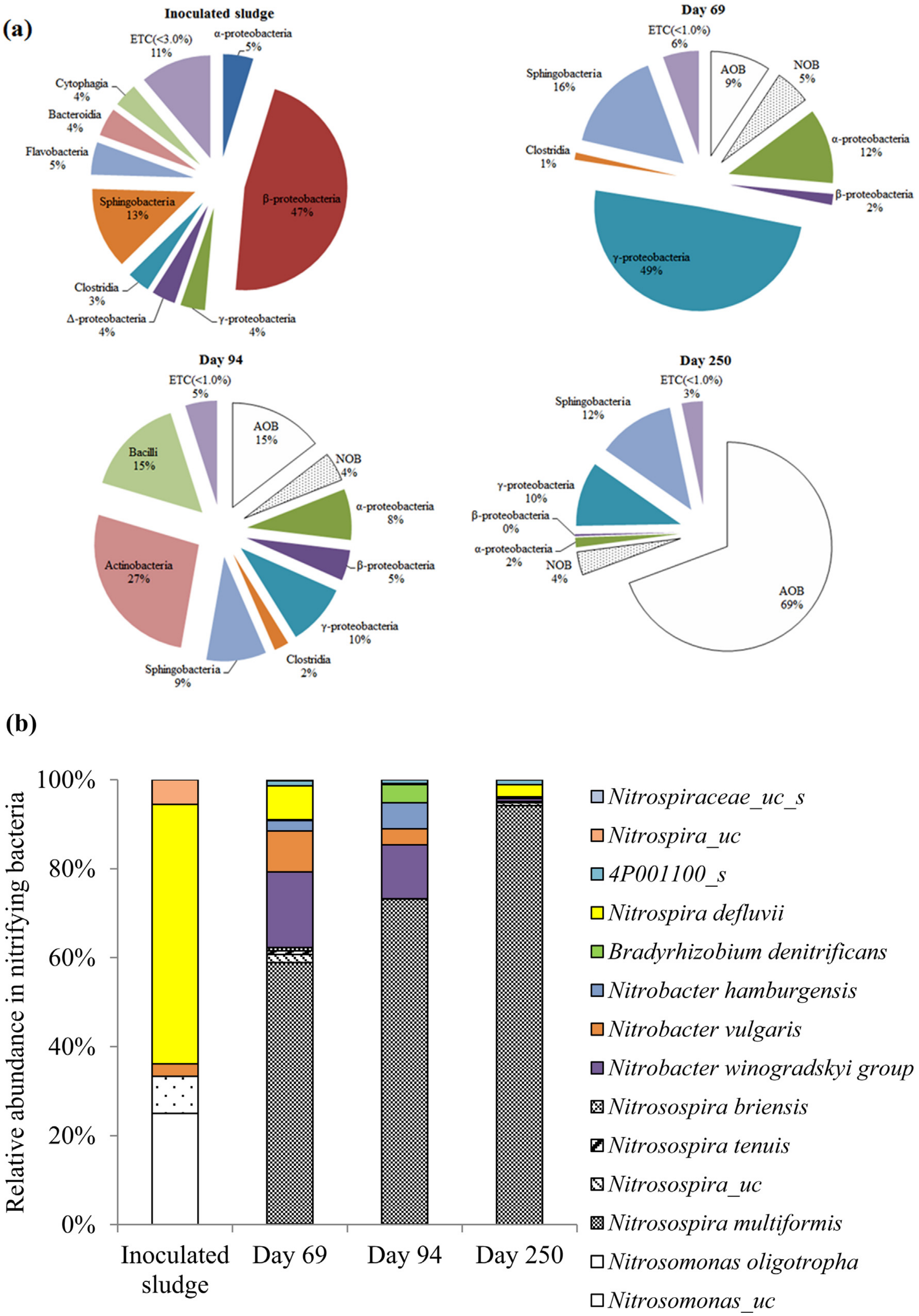

Fig. 2. The distribution of (a) total microbial community in a class level and (b) nitrifying bacteria identified in a species level as ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) in O_2-based HF-MBfR. The patterned bars indicate AOB and colored bars represent NOB. The line is for separating AOB and NOB.(For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
The major enriched species under nitrification condition in HF-MBfR. The most abundant species are bolded and value in parenthesis indicates the percentage fraction of total bacteria numbers.

Nitrification	Day 69	Day 94	Day 250
Ammonia-oxidizing bacteria (AOB)	*Nitrosospira multiformis* (8.6%)	***Nitrosospira multiformis*** (14.5%)	***Nitrosospira multiformis*** (68.6%)
Nitrite-oxidizing bacteria (NOB)	*Nitrobacter winogradskyi* group (2.5%)	*Gordonia sihwensis* (9.1%)	*Nitrospira defluvii* (2.0%)
No related with nitrification	***Rhodanobacter lindanoclasticus*** (27.7%) *Xanthomonadaceae Rudaea* (5.4%)	***Tumebacillus flagellates*** (14.5%)	*Xanthomonadaceae Rudaea* (6.1%)

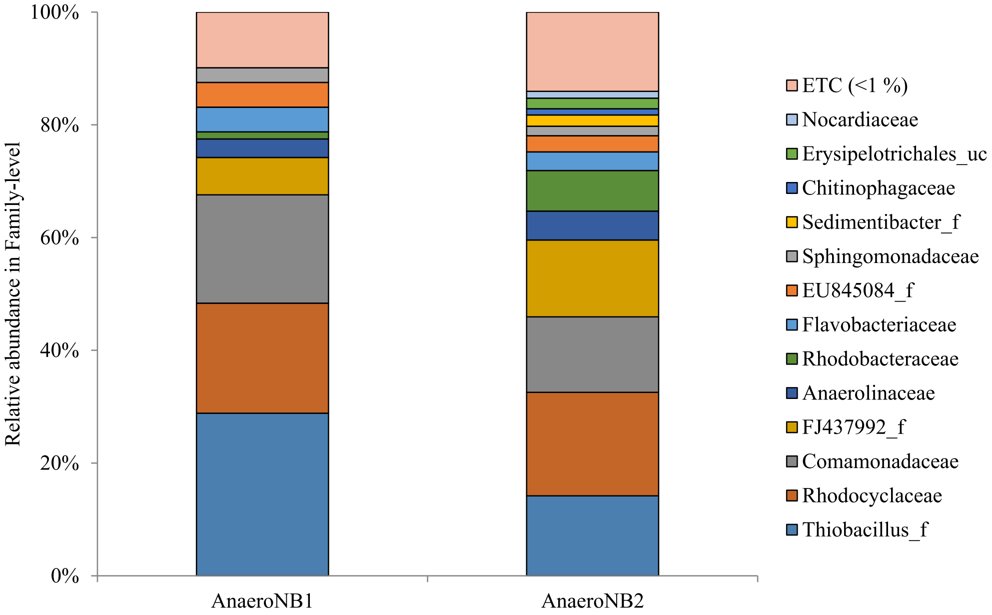

Fig. 3. Family-level bacterial composition in H_2-based HF-MBfR for denitrification.

Table 3
The major bacteria related with denitrification in anaerobic H_2-based HF-MBfR.

Family	Major bacteria in anaerobic H_2-based HF-MBfR
Thiobacillus_f	*Thiobacillus denitrificans* (hydrogenotrophic denitrification) *Thiobacillus thiophilus* (chemolithoautotrophic thiosulfate-oxidizing bacterium)
Rhodocyclaceae	*Azonexus hydrophilus* (heterotrophic denitrification bacteria)
Comamonadaceae	*Hydrogenophaga pseudoflava* (hydrogenotrophic denitrification) *Hydrogenophaga electricum* (hydrogenotrophic denitrification) *Alicycliphilus denitrificans* (facultative heterotrophic denitrification bacteria)
Rhodobacteraceae	*Paracoccus versutus* group (heterotrophic denitrification bacteria)
Anaerolinaceae	*Bellilinea caldifistulae* (strictly anaerobic, filamentous bacteria)

arabinose, a constituent of the bacterial cell wall (Chen et al., 2002). The reason of the occurrence of *Tumebacillus flagellates* could not be clearly determined (Table 2). It has been isolated from the wastewater collected from a casaba starch factory (Wang et al., 2013). The occurrence of heterotrophic bacteria in the late phase could be related to degradation of by-products induced by autotrophs' growth. However, as shown in Fig. 2, the autotrophic nitrifying bacteria quantitatively dominated in the O_2-based HF-MBfR.

3.2. Denitrifying bacteria

As the case of the O_2-based HF-MBfR, the microbial communities of the biomass in the H_2-based HF-MBfR were determined via the pyrosequencing analysis. In general, similar microbial phylum was detected in the biomass, except Chloroflexi, which was detected only in the anaerobic reactor (Fig. S2). Chloroflexi is known as a backbone forming agent (Yoon et al., 2010), and uses biomass-derived carbohydrate to prevent membrane fouling (Miura et al., 2007). Also, it has been reported that Chloroflexi has nirk and nosZ genes related to denitrification (Green et al., 2010). The species richness shown in the rarefaction curve of the anaerobic reactor indicates that a more diverse microbial community structure was developed in the late phase (1237/4.77, Chao/Shannon index) than in the initial phase (1150/4.60). Biomass-derived materials could be used by heterotrophic bacteria like *Chloroflexi*, resulting in a higher microbial diversity.

The six families predominating in the H_2-based HF-MBfR were Thiobacillus, Comamonadaceae, Rhodocyclaceae, Rhodobacteraceae, FJ437992 (belong to the order of Bacteroidales, uncultured), and Anaerolinaceae (Fig. 3). Thiobacillus include hydrogenotrophic denitrifying bacteria such as *Thiobacillus denitrificans*, *Thiobacillus thioparus*, and *Thiobacillus thiophilus*, which were present in the

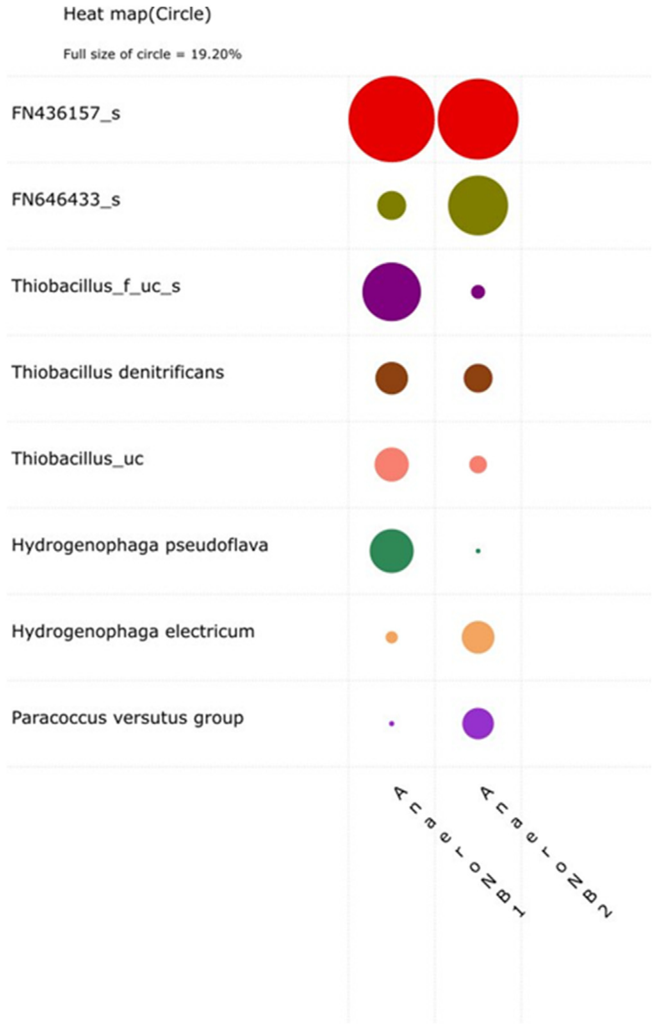

Fig. 4. The heat map represents the composition change in two biomass samples. FN436157_s and FN646433_s belongs to the family of Rhodocyclaceae, Bacteroidales, respectively. The cut-off was 5% for individual sample.

AnaeroNB1 (Table 3). Also, *Hydrogenophaga pseudoflava* (hydrogenotrophic denitrification), *Hydrogenophaga electricum* (hydrogenotrophic denitrification), and *Alicycliphilus denitrificans* (facultative denitrifying bacterium) belonging to Comamonadaceae were also detected. Rhodocyclaeae are denitrifying bacteria preferring oligotrophic conditions (Table 3).

Azonexus hydrophilus, heterotrophic denitrifying bacteria (Reinhold et al., 1986; Chou et al., 2008) were found in the biomass sample collected on Day 250 (denoted as AnaeroNB2) as the main denitrifying bacteria. *Paracoccus versutus* (Rhodobacteraceae) are also a heterotrophic denitrifying bacteria species (Shi et al., 2013). The major species identified as denitrifying bacteria in the AnaeroNB1 and the AnaerobNB2 are summarized in Table 3. Using the heat map analysis, the most abundant species in AnaeroNB1 and in AnaeroNB2 were compared (Fig. 4; 5% cut-off applied for each individual sample). FN436157_s showed 94.8% similarity with *Azonexus hydrophilus* and *Thiobacillus* f_uc_s indicated 94.2% similarity with *Thiobacillus denitrificans*. Overall, hydrogenotrophic denitrifiers (Thiobacillus, Comamonadaceae) were enhanced in AneroNB1, while heterotrophic denitrifiers (Rhodocyclaceae and Rhodobacteraceae) were dominant in AnaeroNB2. Therefore, it was concluded that the shift from autotrophic to heterotrophic denitrification occurred in the H_2-based HF-MBfR. Diversity was increased in the H_2-based HF-MBfR, whereas decreased in the O_2-based HF-MBfR, at the end of the system operation (Table 1). The co-existence tendency of autotrophs and heterotrophs was stronger in the H_2-base HF-MBfR than in the O_2-based one. In the H_2-based HF-MBfR, the appearance of heterotrophic denitrifying bacteria (Fig. 4) could be the reason for the diversity increase. It suggested that a higher nitrogen removal rate might be achieved if the denitrification was operated over a longer time period. In addition, the co-operation of autotrophic and heterotrophic denitrifying bacteria would lead to a resistance to a change of environmental condition, which should be studied as a future research.

4. Conclusions

The analysis of the microbial community structure in the O_2-based HF-MBfR using pyrosequencing showed good enrichment of *Nitrosospira multiformis* as AOB and *Nitrobacter* sp. as NOB. In the case of the H_2-based anaerobic HF-MBfR, hydrogenotrophic denitrifying bacteria were initially dominant. However, over the 250 d operation period, heterotrophic denitrifying bacteria replaced the hydrogenotrophic ones. The heterotrophic denitrification occurred simultaneously with autotrophic nitrate removal at the later phase, utilizing by-products from the autotrophic denitrification in the H_2-based HF-MBfRs. It indicated that more diverse microbial community developed in the system, facilitating stable denitrification.

This study showed the HF-MBfR could be autotrophically operated for an application for treatment of nitrogen-rich wastewater using bubbleless membrane-diffusion and a pyrosequencing technique could investigate the establishment of autotrophic microbial community in HF-MBfR. The microbial community structure was highly enriched with nitrifying/denitrifying bacteria in both O_2- and H_2-based HF-MBfRs within a relatively short period, overcoming a major disadvantage of applying a conventional biofilm reactor for nitrogen removal, which requires a long period for microbial community enrichment in biofilm.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.07.099.

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