

Seasonal Ammonia Removal Performance by Biofilm in a Water Treatment Plant

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Abstract

Nitrogen compounds, represented by ammonia, are one of the most critical pollutants in water bodies because of their role in eutrophication and their toxicity to aquatic organisms. Recently, the biological treatment of ammonia in water resources has been rapidly improved and being applied to numerous water treatment plants, as more and more knowledge has been accumulating on microbial participation of ammonia-utilizing organisms. However, most of the researches on biological removal of ammonia focused on waste water treatment plants (WWTPs) or pilot/laboratory water treatment reactors, where ammonia concentration in the influent waters is usually from 4 to several hundreds even thousands of mg N/L. By far, knowledge of ammonia removal performance with low ammonia load (especially when less than 2.0 mg N/L), such as in drinking water treatment plants (DWTPs), is still limited. Thus in this research, we sampled biofilm from a practical biological DWTP and carried out ammonia removal tests in lab with low ammonia inflow (1.0 mg/L) under dark condition. Our results showed that ammonia can be effectively removed within 2-day incubation when temperature was as low as 11°C, while no significant ammonia removal could be confirmed at 7°C. Molecular analysis showed that canonical ammonia oxidizers (AOA and beta-AOB) may not be responsible for the observed ammonia abatement in the sampled DWTP as well as in laboratory incubation vials. Instead, microbial ammonia assimilation or *comammox* process may be the probable ammonia removal pathway in low ammonia conditions such as DWTPs.

Keywords: ammonia removal, biofilm, biodegradation

INTRODUCTION

Ammonia, a common pollutant in water resources, is mainly derived from the decomposition of nitrogenous organic matters discharged from industrial, agricultural, and domestic wastewater. A high concentration of ammonia in water systems could threaten various aquatic species and affect human health. It can also cause eutrophication in

freshwater and marine water systems. Thus, removing ammonia in water is a vital task in water treatment processes worldwide.

There are several ways to remove ammonia in water, including the ion exchange method, chemical precipitation, adsorption, and biological treatment¹⁾. Among all the listed methods, biological treatment is the only effective way to remove ammonia from water without high-cost maintenance or extra

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chemical addition²⁾. Biofilms, formed naturally in all water environments, allow consistent ammonia removal without high-energy consumption or secondary pollution. And compared to activated sludge treatment, biofilm treatment result in less waste production. These characteristics constitute a simple and reliable method in nitrogen-removal processes in many water treatment plants. The biofilm-based biological treatment can be further divided into aerobic treatment, initialed with nitrification, and anaerobic treatment mainly based on anammox. Compared to a large-scale operation system and strict anoxic-condition maintenance required by anaerobic treatment, aerobic treatment through nitrification can be easily managed and regulated, and thus becomes a cost-effective strategy. Aerobic treatment had been thought to be a two-step nitrification process: ammonia oxidation and nitrite oxidation. And ammonia oxidation is the first and rate-limiting step in nitrification, which is performed by both groups of bacteria and archaea (i.e. ammonia oxidizing beta-proteobacteria (beta-AOB) and ammonia oxidizing archaea (AOA)). Previous studies that analyzed the microbial community in biofilms in various water treatment systems^{3),4)}, with high ammonia load showed the involvement of both nitrifying bacteria and archaea, while the proportions of these two domains of prokaryotes may vary with factors such as inlet ammonia concentration, pH, dissolved oxygen (DO) level, and water temperature^{5),6)}. Recently, *comammox* bacteria (nitrite-oxidizing bacteria *Candidatus Nitrospira*) has been found to be responsible for complete ammonia oxidation to nitrate via nitrite⁷⁾, and can be confirmed in some natural systems such as rivers and lakes⁸⁾. However, their involvement in attached biofilms remains to be fully elucidated. By far, a typical nitrification-based biological water-treatment system is considered to be characterized by increasing nitrite and nitrate concentrations, along with decreasing ammonia concentrations, and being supplied with sufficient dissolved oxygen²⁾.

Till now, both AOA and AOB are reported as responsible ammonia removers in biological wastewater treatment plants (WWTPs),

and considerable knowledge has been accumulated on aspects such as, treatment capability, affiliate water temperatures of biofilm existing in both WWTP and laboratory bioreactor with a high ammonia load (usually 4 to hundreds mg/L of ammonia)^{2),3),9),10)}. However, AOA and AOB were rarely studied in drinking water treatment plant (DWTP) biofilm. As ammonia concentration, a key factor affecting nitrifying microbial community, is at different level in DWTP and in WWTP, microbial composition in DWTP biofilm may be quite different from WWTP biofilm. Moreover, the seasonal fluctuation of influent water temperature in DWTP is more dynamic than in WWTP, as the former receives synchronistic natural water while the latter receives relatively temperature-stable domestic sewage water. Currently, information on seasonal ammonia removal efficiency are restricted to high ammonia condition, while little is known about that under low ammonia concentration. Considering the increasing needs for effective ammonia removal in drinking water resources such as groundwater, it becomes an urgent task to thoroughly evaluate seasonal nitrogen removal performance with low-ammonia inflow, using biofilm retrieved from DWTP. Here, as a fundamental step, we provide a seasonal evaluation of ammonia removal performance using biofilm sampled from a full-scale DWTP which receives raw lake water at near 0.2 mg/L level of ammonia. Seasonal variation in water quality in this DWTP was monitored, and laboratory ammonia removal tests were carried out monthly at *in situ* water temperatures in laboratory. Additionally, existence of AOA and AOB during incubation tests were analyzed using specific molecular techniques. The aim of this study is: 1. evaluate seasonal ammonia removal potential using biofilm retrieved from DWTP; 2. extend the knowledge on low-concentration ammonia removal by biofilm; 3. provide information on temperature limits of biological drinking water treatment technology; 4. clarify the relationship of gene abundance and ammonia removal efficiency.

MATERIALS AND METHODS

Sampling Sampling was conducted

monthly from May 2015 to January 2016 in a biological water treatment plant near Lake Kasumigaura in Tsuchiura City.

Lake water was biologically treated through the untreated water tank, tank A, tank B, and tank C in the water treatment plant (Fig. 1). All water tanks were exposed in outdoor without roofs, and the water temperatures fluctuated seasonally. Each water treatment tank (vertical water depth: 4.75 m) is equipped with about 60% (vol/vol) honeycomb tube (vertical depth: 3m), with a blower settled at the bottom of tank. In this research, approximately 2 L of water was collected monthly for biological analysis. These samples came from the surface water of the untreated water tank, tank A, and tank C (Fig. 1). These water samples were kept in 2 L plastic bottles without bubbles and stored at 4°C until analysis.

Water samples for ammonia removal tests (incubation experiment) were collected from surface water tank A (Fig. 1). These water samples were pre-filtered through GF/F filters (Whatman) and filtered through 0.2 µm pore-size polycarbonate membrane filters (Whatman) to remove microorganisms. The filtered water samples were used as a media base for the following incubation experiments.

Biofilm sample collection Monthly biofilm samples were collected in the manner described in previous study¹²⁾. Briefly, a 13 mm pore size polyvinyl honeycomb carriers were set at 1 m depth in tank A for about one month to accumulate and mature biofilm (Table 1). Subsequently, these polyvinyl honeycomb carriers were retrieved and kept

in a cooler box with tank A water at room temperature until use.

Water quality measurements DO, pH, and chlorophyll-a were measured as described previously¹²⁾. Water samples were first filtered through GF/F filters (Whatman) and then used for further chemical analysis. Ammonia, nitrite, and nitrate ions were measured by indophenol blue, diazotization, and hydrazinium sulfate methods, respectively (n=3).

Laboratory ammonia removal test In order to clarify the monthly difference on ammonia removal potential using biofilm, laboratory incubation experiments were carried out monthly under *in situ* water temperature, using biofilm retrieved from the DWTP on each month. In laboratory scale, the incubation were carried out in 100 mL glass bottles, each of which contained 30 cm² (surface area) of biofilm-covered

Table 1 Honeycomb carrier steeping period for biofilm formation.

	Steeping period	Steeping time (days)
May 2015	4/28 to 5/25	27
June 2015	5/25 to 6/23	29
July 2015	6/23 to 7/22	29
August 2015	7/22 to 8/17	26
September 2015	8/17 to 9/16	30
October 2015	9/16 to 10/16	30
November 2015	10/16 to 11/17	32
December 2015	11/17 to 12/16	29
January 2016	12/16 to 1/18	33

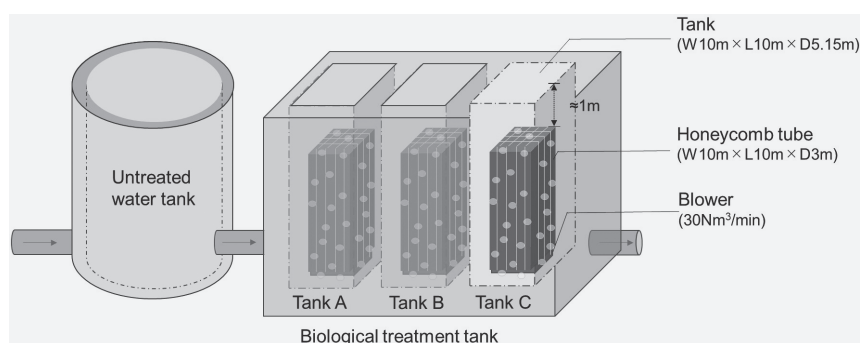


Fig. 1 Schematic diagram of water treatment tank facility at water treatment plant. Arrow shows treatment flow.

honeycomb carrier (or 30 cm² of new honeycomb carrier used as blank control), 19 mL of pretreated water (abovementioned in **Sampling**), and 1 mL of 20 mg/L sodium ammonia solution. The bottles were put in aluminum foil-covered incubators, and all incubation was carried out at *in situ* water temperature condition and shaken at 100 rpm for five days under dark condition. The concentrations of ammonium ions were measured on Day 0, Day 0.5 (12 h), Day 1, Day 2, Day 3, and Day 5 by the methods described above.

Molecular experiments

DNA extraction Biofilm samples (surface area: 4 to 16 cm²) were collected at Day 0 and Day 0.5 during laboratory removal test. These samples were stored in 2 ml microtubes at -80°C before use. DNA were extracted from each sample using ISOIL for Beads Beading DNA extraction kit (Nippon Gene) according to manufactory instruction.

Real-time PCR analysis The abundance of the functional gene *amoA* of thaumarchaeota, betaproteobacteria, as well as the universal 16S rRNA gene of bacteria were determined by real-time PCR assays using specific primers (Table 2). All real-time PCR assays were performed on an Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems). The copy numbers of archaeal *amoA*, beta-peoteobacterial *amoA*, gamma-proteobacterial *amoA* and bacterial 16S rRNA were determined in triplicate on non-diluted samples using either THUNDERBIRD[®] Probe qPCR Mix or THUNDERBIRD[®]

SYBR qPCR Mix (Toyobo) according to manufactory instruction. All reactions were performed in 96-well real-time PCR plates (Applied Biosystems) sealed with optical tape (Applied Biosystems), with each well containing 1 µL of template DNA, 10µL of premix, 0.4 µL of ROX reference dye and primers/probes (each at optimum concentration, varying from 0.15 to 0.3 µM, Table 2). Standards for real-time PCR were generated by using serial dilution of know copies of PCR fragments. To ensure each real-time assay was conducted under high efficiency and specificity, assay validation was performed to optimize primer concentration and annealing temperature. The final efficiency of each real-time PCR assay was from 94.5% to 110.8%.

RESULTS

Water quality of untreated and biologically treated water

Water temperatures were nearly identical in the untreated water tank, tank A, and tank C, and it showed the seasonal change in a typical temperate pattern (Fig. 2a). The highest water temperature was recorded in August as 28.0°C, while the lowest was recorded in January as 7.0°C.

From May to October, pH values fluctuated slightly (6.16 to 7.05), showing no significant difference in the three tanks' water (Fig. 2b). Then, pH values suddenly increased to >9 in November and December (highest in tank C, then tank A and the untreated water tank), and returned to near 8 in January.

Table 2 Primers and probes used in this study.

Target	Gene	Primer/probe	Sequence (5' to 3')	Primer Conc. (µM)	AT [※] (°C)	Ref.
Beta-AOB	<i>amoA</i>	amoA-1F	GGGGTTTCTACTGGTGGT	0.3	58.5	Rotthauwe <i>et al.</i> , 1997 ³³⁾
		amoA-rNew	CCCCTCBGSAAVCCTTCTTC			Hornek <i>et al.</i> , 2006 ³⁴⁾
AOA	<i>amoA</i>	Arch-amoA-for	CTGAYTGGGCTGGACATC	0.15	58.5	Wuchter <i>et al.</i> , 2006 ³⁵⁾
		Arch-amoA-rev	TTCTTCTTTGTTGCCAGTA			
comammox	<i>amoA</i>	Ntsp-amoA 162F	GGATTTCTGGNTSGATTGGA	0.15	48	Fowler <i>et al.</i> , 2018 ²⁷⁾
		Ntsp-amoA 359R	WAGTTNGACCACCASTACCA			
Bacteria	16S rRNA	BACT1369F	CGGTGAATACGTTTCYCGG	0.15	56.5	Suzuki <i>et al.</i> , 2000 ³⁶⁾
		PROK1492R	CGWTACCTTGTTACGACTT			
		Tm1389F	CTTGACACACCGCCCGTC			

※AT: Annealing Temperature

In all months, DO values were highest in tank C (9.7 to 11.1 mg/L), followed by tank A (6.5 to 10.4 mg/L) and the untreated water tank (4.9 to 9.6 mg/L) (Fig. 2c). The monthly dynamics of DO were featured by a slight increase in values from August to January.

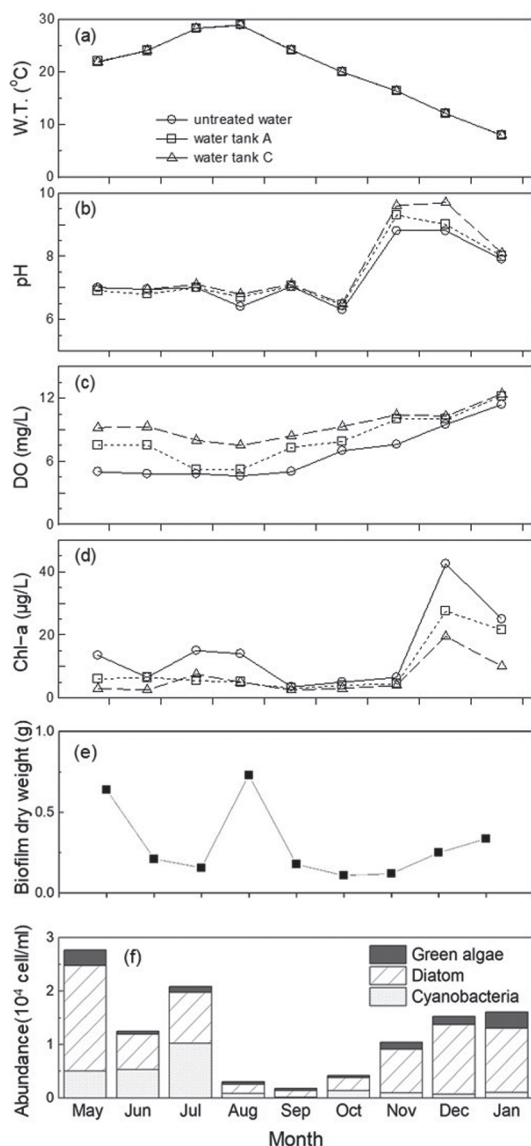


Fig. 2 Monthly changes of physicochemical and biological parameters in untreated water and biologically treated water (tank A and tank C) from May 2015 to Jan 2016. (a): water temperature, (b): pH, (c): DO, (d): chlorophyll-a, (e): dry biofilm, (f): Algae abundance in Kasumigaura Lake.

The chlorophyll-a concentrations ranged from 3.6 to 41.8 µg/L in the untreated water tank, 2.5 to 27.1 µg/L in water tank A, and 2.3 to 19.1 µg/L in water tank C (Fig. 2d). Generally, the chlorophyll-a concentrations became lower along with the treatment flow (from untreated water tank to tank A and to tank C). The values sampled in all tanks kept relatively stable from May to November, with a notable increase in December and January.

The algae composition was not analyzed in this research. For reference only, we collected algae abundances data published on annual water quality report of Kasumigaura Lake to show the seasonal dynamic of algae (note: the sampling station and sampling date were different from this research) (Fig. 2f). As the result, algae abundance were relatively high (>10⁴ cell/ml) in the spring-summer transition time, decreased to low level (at 10³ cell/ml level) in summer, and increased in autumn-winter season (around 10⁴ cell/ml). This seasonal fluctuation in total algae abundance may be affected by the concentrations of dissolved organic compounds, phosphorus and nitrogen compounds from external inflow, and may also due to the geographical and hydrological characteristics of Kasumigaura Lake.

There were no significant seasonal changes in ammonia concentrations over nine months. Highest ammonia values were always recorded in the untreated water tank (ranged from 0.03 to 0.27 mg/L), with a slight decrease in tank A (0.01 to 0.17 mg/L), and more of a decrease in tank C (<0.01 to 0.06 mg/L) (Fig. 3a). Nitrite concentrations were barely detected from May to July, with an increase from August to October, and became barely detectable again from November to January. Taken ammonia concentration in raw water tank as control, the removal efficiency of tank A was calculated as: efficiency (%) = (ammonia conc. in raw water tank - ammonia conc. in tank A) / ammonia conc. in raw water tank, and the removal efficiency of tank A to C was calculated as: efficiency (%) = (ammonia conc. in raw water tank - ammonia conc. in tank C) / ammonia conc. in raw water tank. The results showed that ammonia were gradually removed from

tank A to C, and from 61.8% to 100% of ammonia can be removed after treatment in tank A to C when temperature was $\geq 11^{\circ}\text{C}$ (from May to December).

Similar to ammonia concentration, nitrite concentrations were also always highest in the untreated water tank (0.03 to 0.16 mg/L), then tank A (0.02 to 0.09 mg/L) and tank C (0.01 to 0.03 mg/L) (Fig. 3c). In contrast, nitrate concentrations were generally highest in tank C (0.17 to 0.84 mg/L), then tank A (0.17 to 0.64 mg/L) and the untreated water tank (0.15 to 0.68 mg/L) (Fig. 3d). The seasonal change in nitrate concentrations was characterized by higher values from August to October, with a peak in October. Considering the DO concentrations together, these results indicated ammonia was, or at least partially removed through aerobic degradation via the water treatment flow (from the untreated water tank to tank C).

Laboratory ammonia removal test In

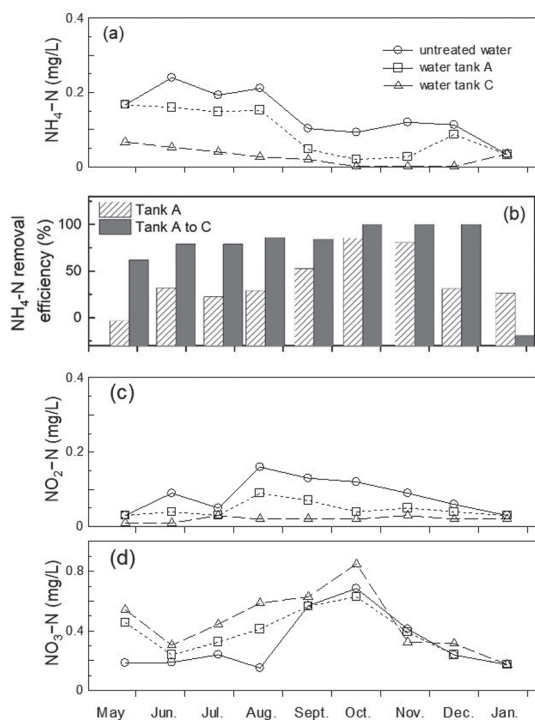


Fig. 3 Monthly changes of nutrients concentration in untreated water and biologically-treated water (water tank A and water tank C) from May 2015 to Jan 2016. (a): ammonia, (b) ammonium removal efficiency (c): nitrite, (d): nitrate.

order to determine the monthly ammonia removal rates with low ammonia inflow, laboratory incubation experiments were carried out at *in situ* water temperature monthly from May 2015 to January 2016. As a result, the changes in the ammonia concentration during the 5-day incubation period are shown in Fig. 4.

From May to September (WT: 22 to 28°C), an average of 90.7% of ammonia was removed within 12h; while from October to December (WT: 11 to 20°C), only 47.9% of ammonia were removed within 12h, indicating that ammonia removal by biofilm requires longer retention time when the temperature was lower than 20°C . From October to December, despite the relatively lower removal rates in the first 12 h, 97.5% of ammonia was removed after 48h incubation, showing the activity of biofilm was partially inhibited when the water temperature was from 11 to 20°C . In December (WT: 7°C), ammonia concentrations increased with incubation time in the biofilm-addition group. The increase in ammonia concentrations may be considered as the result of natural degradation of organic compounds (ammonification) existing in the biofilm exceeded ammonia oxidation (or ammonia consumption) in the biofilm, when the temperature was as low as 7°C .

Using the data from Day 0 to Day 2 of the ammonia removal test, the ammonia removal rate per surface area of the honeycomb carrier was calculated for each month (Fig. 5). The removal rates showed seasonal characteristics: higher rates were confirmed from May to August, and lower rates were observed from December to January. Ammonia removal rate shifted from high to low during autumn-winter transition period (October to November).

Real-time PCR results The abundance of archaeal *amoA* (represented for AOA), beta-proteobacterial *amoA* (represented for beta-AOB), and bacterial 16S rRNA gene were analyzed on raw biofilm (Day 0 samples) and Day 0.5 incubation samples via real-time PCR. The results were demonstrated as the copy number per biofilm surface area in Fig. 6. As the result of bacterial 16S rRNA, the copy number in Day 0 samples ranged from 4.3×10^3 (September)

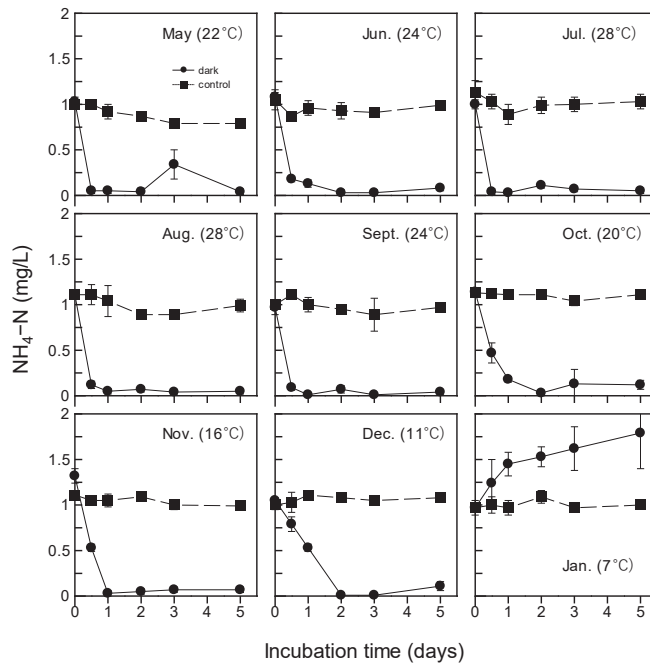


Fig. 4 Ammonia removal by biofilm at in situ water temperature (May 2015 to Jan 2016).

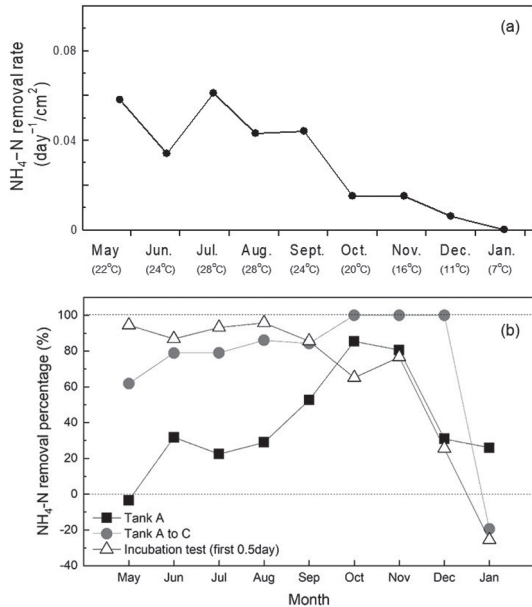


Fig. 5 Monthly changes of ammonia removal by biofilm. (a. ammonia removal rate in the first 0.5 day in incubation experiments. Each temperature indicates incubation temperature, which is the same as water temperature at the sampling time. b. ammonia removal percentage in water treatment plant and incubation experiments).

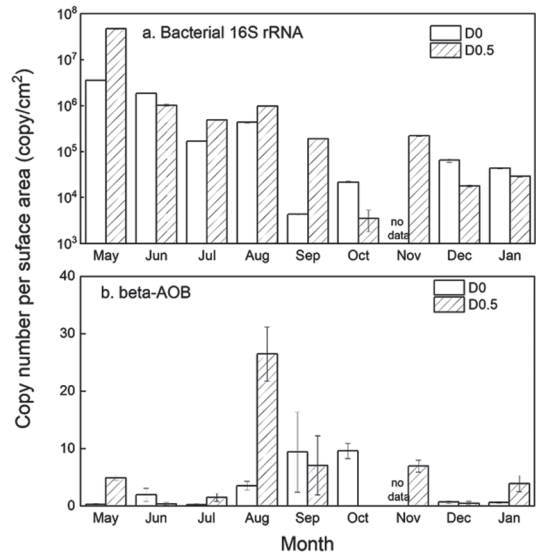


Fig. 6 qPCR results of gene copy numbers (per surface area of biofilm) in Day 0 and Day 0.5 samples (a: bacterial 16S rRNA, b: beta-AOB).

to 3.5×10^6 (May) copies per cm^2 , with higher copy number detected in May to August. Comparing the abundance in Day 0 and Day 0.5, significant increase can be recorded in May and September, while the copy number stayed at same level or slightly decreased after 0.5-day incubation. As the result of *amoA* gene, in all samples, no archaeal *amoA* copy can be detected. For the bacterial counterpart, the copy number of beta-proteobacterial *amoA* was detectable, but stayed at extremely low level (generally less than 10 copies per cm^2 , except for Day 0.5 in August, which is 26.5 copies per cm^2 in average) in all samples. Considering even the highest beta-proteobacterial *amoA* (ie. D0.5 in August) only share 0.002% of total bacterial 16S rRNA, beta-AOB may only play as minority contributor in ammonia removal in this research. These results showed that, although nitrification was thought to be the main principle to remove ammonia in the sampled DWTP, traditional ammonia oxidizers (AOA and AOB) may not significantly contribute to low-concentration ammonia removal in DWTPs.

DISCUSSION

Temperature is an important factor affecting the removal efficiency of biofilm. Previous researches have shown that temperature significantly affects the degradation rates of several substances such as musty odor compound (geosmin)¹¹ and petroleum hydrocarbon¹², as low temperature may inhibit microbial growth by changing favorable substrate affinity for microbes¹³. Ammonia, which has a much simpler structure and lower molecular weight than those refractory substances, can be effectively and rapidly removed through aerobic nitrification by biological treatments such as biofilm and activated sludges (both contain responsible nitrifying prokaryotes). The optimum temperature for the growth of majority of nitrifying bacteria was 30 to 35°C^{14, 15}. When temperature is lower than 15°C, the activity of nitrifying bacteria dropped remarkably, leading to a sharp decrease in nitrification rates^{14, 16, 17}. The effect of seasonal variations, or water temperature, were studied mainly under high

ammonia load condition in previous studies. For instance, ammonia removal rates decreased from 83% to 54% when water temperature was set from 13°C to 6.5°C using biological aerated filter when initial ammonia concentration was 25 mg/L¹⁸. Another study using an upflow biological aerated filter showed nitrification may be impacted when temperature was below 14°C with ammonia load of 1500 mg/L¹⁹. When temperature was 6°C, according to Sudarno et al.¹⁴, ammonia oxidation was completely prevented in a immobilized biofilm system treating saline waters (initial ammonia concentration: 60 mg/L), but could be fully recovered when incubation temperature was shifted to room temperature. At low ammonia level,²⁰ (Liu et al. observed an average of 92.6% in summer and an average of 77.5% in winter (temperature ranged from 5.1°C to 8.1°C) with ammonia influent at around 2.0 mg/L level in a continuously operated system using enriched lab scale bioreactor, which is so far the only evidence we can find about low-concentration ammonia removal under temperature dynamics.

This research is different from previous researches, as the treatment is based on naturally formed biofilm retrieved monthly from a full-scale DWTP, and the incubation test was carried out at low ammonia level (which is 1.0 mg/L) without extra nutrients additions in a microcosm-like system instead of long term operated bioreactor. Our results showed that low-concentration ammonia (initial ammonia concentration: 1.0 mg/L) can be fully removed within 2-day incubation when the temperature was as low as 11°C, while no significant ammonia removal could be confirmed at 7°C. It should be noted that the ratio of oxygen to ammonia is also an important index in biological ammonia removal technologies. Heavy ammonia load accompanied by low oxygen supplement, especially when the ratio is lower than 2 g O_2/g ammonia, may cause a significant decrease in ammonia removal rate^{21, 22}. In contrast, this ratio kept above 5.0 g O_2/g ammonia in the treatment process in the sampled DWTP, which may help to achieve effective ammonia removal at the temperature as low as 11°C. This indicates that biofilm

under sufficient aeration may be a competitive option for drinking water treatment (where ammonia concentration is usually below 2.0 mg/L) in tropical to subtropical-temperate countries where winter temperatures are higher than 10°C.

The failure of the detection of AOA and AOB in biofilms raised the question that whether ammonia oxidation (via canonical AOA and AOB) are the probable pathway for the recorded ammonia decrease in the DWTP in this research. Although both AOB and AOA have been detected at ubiquitous level by molecular methods (in most cases, from 10^3 to 10^9 *amoA* copies per g sludge²³⁾ in biological treatment processes such as activated sludge, biological aerated filter (BAF), granular activated carbon (GAC) etc., all these systems are typical with high ammonia concentrations (from tens to thousands mg/L levels). Existence of AOA and AOB has been reported in the water phase in a drinking water distribution system and ground water²⁴⁾ and numerous natural water systems (freshwater and oceans), some of which are at similar ammonia level to the raw water source in the DWTP in this research. However, different from free-living microorganism existing in the water phase, immobilized biofilm has multiple layer structure on solid surface, which form an interactive and stable micro-ecosystem consisting of various species of microorganisms²⁵⁾. As ammonia is a competition resource in water environment for various species including algae, hetero- and autotrophic prokaryotes, and considering the low ammonia concentration in both raw water (around 0.2 mg/L) and incubation vials (1 mg/L), biofilm may not be an advantage for the immobilization of AOA and AOB in the community. Similarly, AOA were at detectable level in Granular activated carbon after 3-week enrichment (after stopping prechlorination) with 5 mg $\text{NH}_4^+\text{-N/L}$ supplement, but both AOA and AOB were not detectable when $\text{NH}_4^+\text{-N}$ was under 0.4 mg/L²⁶⁾. In another study with similar ammonia level (from 0.75 to 1.03 mg $\text{NH}_4^+\text{-N/L}$)²⁷⁾, no AOA nor AOB-like species could be confirmed by denaturing gradient gel electrophoresis analysis after 180 days pilot-scale enrichment on

Granular activated carbon. Instead, bacterial community contained Nitrospira-like species (nitrite-oxidizing bacteria) were confirmed. Moreover,²⁸⁾ Fowler et al. 2018 revealed that, the newly-confirmed^{7), 29), 30), 31)} complete ammonia oxidizer: *comammox* Nitrospira were the dominant ammonia oxidizers in biofilms of a rapid sand filtration system in a DWTP, make up about 10% of total bacteria (16S rRNA copies), while AOB were at low levels and AOA were undetectable. Based on these facts, the existence of *comammox* bacteria were checked in our samples. Since the samples on D0 and D0.5 were not enough to perform extra qPCR (*annot.* several tests have been conducted to check the assay accuracy), the remaining samples on D2 (6 samples on Day 2 from July to December) were analyzed by qPCR using primer set Ntsp-*amoA* 162F and Ntsp-*amoA* 359R²⁸⁾, which targets the *comammox amoA* in both clade A and clade B *comammox* Nitrospira. In these samples, *comammox* were detected from 6.1 to 103.9 copies/cm² (average: 32.8 copies/cm²), outcompeted canonical AOB (which were detected from 0.3 to 59.5 copies/cm², average: 13.6 copies/cm²). And still, AOA were not detectable. The result that *comammox* were more abundant than canonical AOA and AOB, shows that *comammox* may be more important than AOA and AOB in immobilized biofilm with low ammonia income. However, the ratio of *comammox* Nitrospira to total bacteria (16S rRNA copies) in this research were from 0.02% to 0.3%, which is much lower than that in²⁸⁾ Fowler et al. 2018's samples, indicating that *comammox* may only contribute to a portion of ammonia removal in this research. And according to³²⁾ Roots et al. 2019, who found *comammox* was the dominant ammonia oxidizer in a mainstream nitrification reactor, low N concentration and low DO concentration would be potential niche segregation factor of *comammox* bacteria. Here we suspect, the environment in biofilm with low N concentration and high DO concentration may not be beneficial for the growth for *comammox* (as well as for AOA and AOB). Considering that ammonia assimilation (uptake) by heterotrophic bacteria may contribute to about 30% of

ammonia reduce in natural waters³³), here we suggest, the ammonia removal observed in the DWTP as well as in incubation experiments in this research may be the result of total microbial community or *comammox* bacteria, instead of canonical AOA or AOB.

The molecular analysis in this research were showed as copy numbers of specific genes measured by real-time PCR. However, abundances (gene copy number) of certain species can not reflect the whole microbial community, and the design of primer sets sometimes cause to statistical bias in the final results. To gain further information of microbial composition and their potential functions in biofilms in DWTPs, next generation sequencing analysis would be a powerful tool and is required in further studies. In addition, it should be noted that our results can only represent characteristics of biofilm formed in immersion filtration systems (ie. Honeycomb tube settled in tanks with aeration systems, low ammonia, high DO). Since environmental factors should be largely different in water treatment systems such as rotating biological contactor (RBC), and biological contact filtration (BCF) etc., dominant microorganism and their performance in such systems need to be fully understood.

CONCLUSIONS

In this research, we showed that ammonia in raw lake water had been biologically removed by the biofilm treatment process from tank A to tank C in the DWTP facility. Different from wastewater treatment facilities, the DWTP in this research was featured with low ammonia concentration (around 0.2 mg/L) in raw water, and high DO (consistent aeration from bottom) in treatment process. As the result, by monitoring water quality in the DWTP each month, we found that ammonia can be effectively removed through tank A to C from May to December, but the removal efficiency sharply decreased in January. Based on vial-incubation test in laboratory, we found that ammonia could be rapidly removed within 12h from May to September, while it took twice as the time to fully remove ammonia from October to

December. The removal rates shifted during the autumn-winter transition period (October to November), and negligible ammonia removal could be confirmed in December's test. The molecular analysis on *amoA* and 16S rRNA gene in the biofilm samples on Day 0 and Day 0.5 showed that traditional AOA and AOB (ca. beta- subclass) were barely detectable, indicating that canonical ammonia oxidizers may not be responsible for the ammonia removal in the DWTP as well as in vial-incubations. And this would be because immobilized biofilm structures under low ammonia concentration may not be ecologically beneficial for the growth of AOA and AOB. Moreover, although *comammox* outcompeted AOB and AOA in Day 2 samples, they only shared about 0.02 to 0.3% of total bacterial cells. Here we suggest that *comammox* may contribute to a portion of ammonia removal, while ammonia assimilation (or uptake) by heterotrophic microorganisms may play a major role in such biofilm structure under low ammonia and high DO condition.

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