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Ammonia half-saturation constants of sludge with different community compositions of ammonia-oxidizing bacteria

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ABSTRACT

Owing to the kinetic differences in ammonia oxidation among ammonia-oxidizing microorganisms (AOM), there is no standard set of kinetic values that can be used as a representative set for nitrifying wastewater treatment plant (WWTP) design. As a result, this study clarified a link between the half-saturation constants for ammonia oxidation (*Ks*) and the dominant ammonia-oxidizing bacterial (AOB) groups in sludge from full-scale WWTPs and laboratory-scale nitrifying reactors. Quantitative polymerase chain reaction analyses revealed that AOB affiliated with the *Nitrosomonas oligotropha* cluster were the dominant AOM groups in the sludge taken from the low-ammonia-level WWTPs, while AOB associate with the *Nitrosomonas europaea* cluster comprised the majority of AOM groups in the sludge taken from the high-ammonia-level WWTPs and nitrifying reactors. A respirometric assay demonstrated that the ammonia *Ks* values for the high-ammonia-level WWTPs and nitrifying reactors were higher than those of the low-ammonia-level plants. Using the *Ks* values of available AOM cultures as a reference, the *Ks* values of the analyzed sludge were mainly influenced by the dominant AOB species. These findings implied that.different sets of kinetic values may be required for WWTPs with different dominant AOM species for more accurate WWTP design and operations.

Keywords: Ammonia-oxidizing bacteria, Ammonia half-saturation constant, Wastewater treatment plant

1. Introduction

An effective design and operation of nitrification process requires a better understanding of the microbial compositions and kinetics of nitrifying microorganisms. For common design procedure of nitrifying wastewater treatment plants (WWTPs), a design aerobic sludge residence time (SRT) is determined based upon the kinetics of ammonia -oxidizing microorganisms (AOMs) which is usually the rate-determining-step microorganisms of the system. Numerous studies revealed that the communities of AOM species in WWTPs were generally selected by wastewater characteristics, system configuration and operational practices [1-4]. Moreover, the ammonia half-saturation constant (K_s), one of the main kinetic parameters used to determine the design SRT for nitrifying WWTP, was found to be distinct among AOM species. For example, the ammonia K_s values of ammonia-oxidizing archaea (AOA) are in the range of ~1-10 μgN/L for ammonia oxidation, while those of ammonia-oxidizing bacteria (AOB) are in the range of ~1-100 mgN/L [5]. For AOB, the ammonia K_s values are also different appreciably among distinct phylogenetic groups of microorganisms. For example, members of the *Nitrosomonas oligotropha* and *Nitrosospira* clusters, AOB with high affinity to ammonia, have ammonia K_s values lower than those of AOB with low affinity to ammonia, such as members of the *Nitrosomonas europaea* cluster [5]. As a result, neither set of kinetic values should be commonly used to determine a design SRT for all nitrifying WWTPs. The concern of AOM community composition data prior to the selection of kinetic values should be considered for effective design and operation of nitrification process.

In this study, the ammonia K_s was selected as the initial kinetic parameter of interest to elucidate the ammonia K_s of mixed AOM species. This study aimed to investigate the link between the dominant AOM species and the ammonia K_s values of sludge taken from full-scale WWTPs and laboratory-scale nitrifying reactors. Because the involvement of AOA in ammonia oxidation in WWTPs is still unclear, this study focused only on the systems with AOB dominance.

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2. Materials and Methods

2.1. Sludge Samples

Sludge samples were collected from three full-scale WWTPs (A, B, and C) and laboratory-scale nitrifying reactors (D and E). All WWTPs are activated sludge systems. The WWTPs had different wastewater characteristics and treatment efficiencies. Plants A and B received industrial wastewater, while domestic wastewater was fed into plant C. Samples were collected from plants A and C in two time points, within a period of six months (A-1, A-2, C-1, and C-2) to examine the consistency of the results at different time points. Wastewater characteristics (i.e., BOD and ammonia concentration) of plants A, B, and C are shown in Table 1. At each plant, equal volumes of sludge were taken from four sampling locations in an aeration tank. The samples were mixed before being kept on ice during transportation.

The laboratory-scale nitrifying reactors were operated in fill-and-draw mode with seed sludge from a full-scale municipal WWTP. The reactors were supplied with an inorganic medium containing ammonium concentrations of 238-290 and 435-500 mgN/L for reactors D and E, respectively (Table 1). Both reactors were operated with hydraulic retention time (HRT) of 5 days and SRT of 7 days for 6 weeks. At steady state condition, the ammonia concentrations in effluent were 75-100 and 168-171 mgN/L for reactors D and E, respectively. The inorganic medium (per liter) contained NH_4Cl, NaCl (0.5 g), $MgCl_2 \cdot 6H_2O$ (0.4 g), $CaCl_2 \cdot 2H_2O$ (0.1 g), KCl (0.5 g), KH_2PO_4 (0.2 g), H_3BO_3 (0.03 g), $MnCl_2 \cdot 4H_2O$ (0.1 g), $CoCl_2 \cdot 6H_2O$ (0.19 g), $NiCl_2 \cdot 6H_2O$ (0.024 g), $CuCl_2 \cdot 2H_2O$ (0.002 g), $ZnSO_4 \cdot 7H_2O$ (0.144 g), $Na_2MoO_4 \cdot 2H_2O$ (0.036 g), 8 ml of HCl (12.5 M), 1 mL of nonchelated trace element mixture [6], 1 mL of vitamin solution [6], 1 mL selenite-tungstate solution [6], and bicarbonate solution. The pH was adjusted to 7.2-7.8 using NaOH.

2.2. Quantitative Polymerase Chain Reaction (qPCR)

Numbers of AOM in each sludge sample were determined using qPCR technique. DNA was extracted from 2 mg dry weight of sludge using the Fast-DNA SPIN kit for soil (QBiogenes, USA). For each sludge sample, duplicate sets of extracts were prepared. From each set of extracts, two extracts were pooled to minimize bias. QPCR analysis was separately conducted for the duplicate sets of extracts, each with three 10-fold dilution series in duplicate, using a Light Cycler 480 instrument (Roche, Germany) with a Maxima SYBR Green/ROX QPCR Master Mix (Fermentas, USA). AOA *amoA* genes were enumerated using the primers Arch-*amo*AF and Arch-*amo*AR [7]. The quantification of the AOB *amoA* genes was performed using the primers *amo*A1F and *amo*A2R [8]. To calculate the cell numbers of AOB from the AOB *amoA* gene numbers, the conversion ratio of 2.5 was used, as described by Norton et al. [9]. The primers amoNo550D2f and amoNo754r [2] were used to quantify the *amoA* genes of the *N. oligotropha* cluster. The 16S rRNA genes of *N. europaea* cluster were quantified using the primers NSMeur-828F and NSMeur-1028R [10]. The probe match function in the ARB program package (version 2.0; Department of Microbiology, Technische Universitat Munchen [http://www.arb-home.de]) and the SSU rRNA database suggest that this primer set likely includes all sequences of *N. europaea*, *Nitrosomonas eutropha*, and *Nitrosomonas halophila*, but not *Nitrosomonas mobilis* in the amplification. For comparison, the abundance of various AOB species contained in sludge sample, the numbers of gene copies among total AOB, the *N. oligotropha* and the *N. europaea* clusters were converted to cell numbers on the basis of the numbers of the genes found in one cell. The cell numbers of the *N. oligotropha* cluster were calculated using the conversion ratio of 2.5 [9]. Because one AOB cell contains one copy of the 16S rRNA gene, *N. europaea* cluster cell numbers were equivalent to the number of 16S rRNA genes that were quantified [11]. Details for qPCR thermal profile of each primer set are shown in Table 2.

Table 1. Wastewater Characteristics

WWTP/Nitrifying reactor	Parameters			
	BOD in influent (mg/L)	BOD in effluent (mg/L)	NH_3 in influent (mgN/L)	NH_3 in effluent (mgN/L)
WWTP A	1,800-2,150	8-12	173-360	13-20
WWTP B	187	2	36	2-12
WWTP C	47-59	8-9	7	1-2
Nitrifying reactor D	-	-	238-290	75-100
Nitrifying reactor E	-	-	435-500	168-171

Table 2. Primers for Polymerase Chain Reaction Amplification

Primer	Target gene	Standard DNA	Condition	Reference
Arch-*amo*AF Arch-*amo*AR	AOA *amoA* genes	clone AOA-S-4 (GQ390338)	95°C for 10 min, followed by 40 cycles in 60 s at 95°C, 60 s at 56°C and 30 s at 72°C, and data captured for each cycle at 78°C for 15 s	[7]
amoA1F amoA2R	AOB *amoA* genes	*N. europaea*	95°C for 10 min, followed by 40 cycles in 60 s at 95°C, 60 s at 56°C and 30 s at 72°C, and data captured for each cycle at 78°C for 15 s	[8]
amoNo550D2f amoNo754r	*N. oligotropha* cluster *amoA* genes	*N. oligotropha*	95°C for 10 min, followed by 40 cycles in 30 s at 95°C, 60 s at 56°C and 60 s at 72°C, with data captured for each cycle at 78°C for 15 s	[2]
NSMeur-828F NSMeur-1028R	*N. europaea* cluster 16S rRNA genes	*N. europaea*	95°C for 10 min, followed by 40 cycles in 10 s at 94°C, 30 s at 60°C and 60 s at 72°C, with data captured for each cycle at 78°C for 15 s	[10]

2.3. Respirometric Assay

The ammonia K_s and the maximum specific oxygen uptake rate ($SOUR_{max}$) of each sludge sample were determined using respirometers. The sludge was washed three times with the inorganic medium containing no ammonia to remove residual ammonia from the sludge. The washed sludge was then transferred to 250 ml bottles and closed. Each bottle was equipped with a DO probe (Cellox 325 DO probe, WTW, Germany) connected to a DO meter (Oxi340i meter, WTW, Germany) for a final concentration of 1,000 mg MLSS/L (for sludge from the full-scale WWTPs) or 100 mg MLSS/L (for sludge from the laboratory-scale nitrifying reactors). The assay was carried out by varying the ammonia concentrations from 0 to 400 mgN/L in the inorganic medium. To determine the oxygen consumption of heterotrophs, a negative control without ammonia addition was conducted in parallel. Allythiourea (ATU) at a final concentration of 10 mg/L was also added into the negative control to inhibit ammonia monooxygenase of AOB [12]. For all experiments, sodium azide (NaN_3) at a final concentration of 24 μm was added to inhibit the activity of nitrite-oxidizing bacteria [13]. For each run, the oxygen uptake was on-line recorded per minute by a multi-lab pilot v5.06 software (WTW, Germany). The *SOUR* was calculated from the slope of oxygen depletion curve. The ammonia K_s and $SOUR_{max}$ were determined by fitting the *SOUR* and initial ammonia concentrations to the Michaelis-Menten equation (Eq. (1)) with the assistance of version 11.0 of the SigmaPlot program.

$$SOUR = \frac{SOUR_{max}[S]}{K_s + [S]} \tag{1}$$

Where *SOUR* is the specific oxygen uptake rate (mgO_2/(mgMLSS-hr)), $SOUR_{max}$ is the maximum *SOUR* (mgO_2/(mgMLSS-hr)), [S] is the ammonia concentration (mgN/L) and K_s is the ammonia half-saturation constant (mgN/L).

3. Results and Discussion

3.1. Numbers and Community Compositions of Ammonia-Oxidizing Bacteria

In this study, total AOA *amoA*, AOB *amoA*, *N. oligotropha amoA*, and *N. europaea* 16S rRNA genes were analyzed by qPCR technique. The AOA *amoA* primers confirmed that we selected only the sludge samples with AOB dominance, confirmed by undetectable of AOA *amoA* genes (the limit of detection (LOD) = 5 × 10^3 copies/mgMLSS). For comparison the gene copies among total AOB, the *N. oligotropha* and the *N. europaea* clusters, the gene numbers were converted to cell numbers on the basis of the numbers of the genes found in one cell (Fig. 1). The resulting qPCR demonstrated that AOB affiliated with the *N. europaea* cluster comprised the majority of the AOB consortium in WWTPs having high residual ammonia and nitrifying reactors (sludge A, D, and E). The influent ammonia concentrations of plant A and reactors D and E were in the range of 173-500 mgN/L and the effluent ammonia concentrations were in elevated levels of 13-171 mgN/L. In contrast, members of *N. oligotropha* were the dominant AOB in sludge from WWTPs with

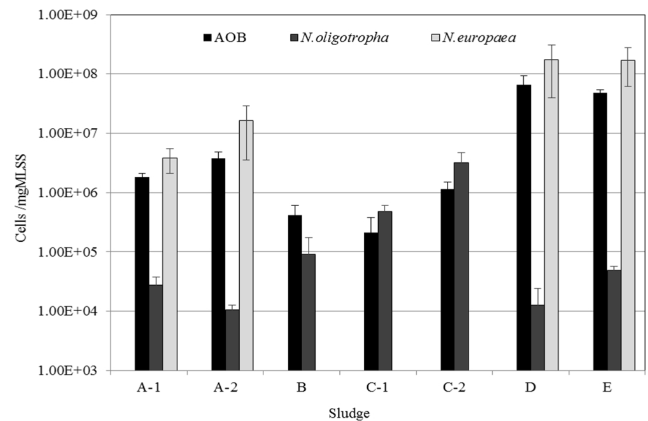

Fig. 1. Numbers of AOB, *N. oligotropha*, and *N. europaea* in sludge of full-scale wastewater treatment plants and nitrifying reactors.

low residual ammonia (sludge B and C). The influent ammonia concentrations of plants B and C were in the range of 7-36 mgN/L which was considerable lower than plant A and reactors D and E. The effluent ammonia concentrations were also in the low levels of 1-12 mgN/L. The variation of the dominant groups of AOB (*N. oligotropha* vs *N. europaea* clusters) among the full-scale WWTPs and nitrifying reactors can be explained by the ammonia affinity of each AOB group. Members of the *N. oligotropha* and *Nitrosospira* clusters, which are AOB with high ammonia affinity, have ammonia K_s values ranging from 0.42 to 4.47 mgN/L. While AOB with low ammonia affinity, the *N. europaea* cluster, have a higher range of the ammonia K_s values, between 1.62 and 111.16 mgN/L (Fig. 3). The variation in ammonia-like habit of AOB leads to the appearance of distinct AOB groups in different habitats. *N. oligotropha* and *Nitrosospira* clusters were found to be common in low-ammonia-level WWTPs [2-3, 14], while members of *N. europaea* cluster were often found in high-strength ammonia WWTPs [15-16].

3.2. Linking between the Ammonia K_s and the Community Compositions of AOB

The combinations of qPCR and respirometric assays were conducted to reveal the relationship between the dominant AOM communities and their ammonia K_s. Respirometric assay was performed under various ammonia concentrations. The specific oxygen uptake rate ($SOUR_{max}$) and the ammonia K_s were calculated for each treatment (Fig. 2). The $SOUR_{max}$ values of full-scale WWTP sludge were in the range of 5.15 to 9.42 × 10^{-3} mgO_2/(mgMLSS-hr). The $SOUR_{max}$ values of the nitrifying sludge were in the range of 6.01 to 32.52 × 10^{-3} mgO_2/(mgMLSS-hr). The ammonia K_s values for sludge A, D, and E were higher than those of the other sludge (Fig. 2).

In order to investigate the relationship between the ammonia K_s values and the dominant AOM species in the sludge samples, the ammonia K_s values of AOM cultures and enrichment were compared with those of sludge samples (Fig. 3). Only AOB related to the *Nitrosospira*, *N. oligotropha*, and *N. europaea* clusters were included because they were the most commonly detected AOB in WWTPs [1-3, 14]. The ammonia K_s values of AOA cultures and enrichment were modified based on a review by Limpiyakorn et al. [5]. The results showed that the ammonia K_s values of all

Fig. 2. Specific oxygen uptake rate (SOUR) under various ammonia concentrations.

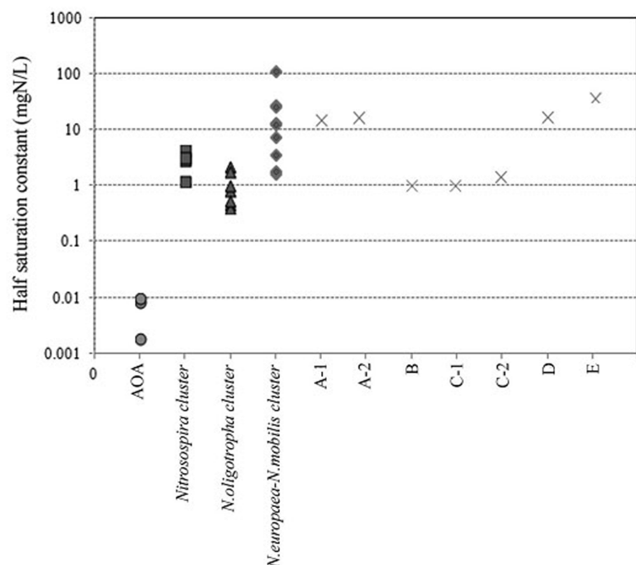

Fig. 3. Ammonia K_S values of AOM cultures (modified from [5]), sludge from full-scale wastewater treatment plants and nitrifying reactors.

sludge samples were in the range of the ammonia K_s values of the three representative AOB clusters.

For ammonia K_s values of sludge A, including A-1 and A-2, which was previously acclimatized with high ammonia were in the range of the ammonia K_s values of the *N. europaea* cluster (Fig. 3). The ammonia K_s results agreed with the specific qPCR results, showing the dominance of the *N. europaea* cluster in plant A of both time points. As with sludge A, the ammonia K_s values and the dominant AOB species detected in the nitrifying reactors with high ammonia levels (D and E) were related to the *N. europaea* cluster. While for the sludge acclimatized with lower ammonia (B and C), the ammonia K_s values of all the sludge samples were close to those belongs to the *N. oligotropha* and *Nitrosospira* clusters (Fig. 3) which was identified to be the dominant AOB species.

3.3. Are Different Sets of Kinetic Values for Ammonia Oxidation Required for the Design and Operation of WWTPs with the Mixture of AOM Species?

This study indicates that sludge with different AOB community compositions exhibits distinct ammonia K_s values. It has been

reported that other kinetic parameters (i.e., μ_{max}, Y, and k_d) for ammonia oxidation also differ among AOM groups. For example, the maximum specific growth rate (μ_{max}) of the AOB affiliated with the *N. europaea* cluster was higher than that of AOB related to the *Nitrosospira* and *N. oilgotropha* clusters, including previously reported AOA [5]. This suggests that for WWTPs with different AOM dominant species, specific sets of kinetic values for ammonia oxidation should be applied for more accurate plant design and operation. That said the kinetic values of sludge with low-ammonia affinity AOB dominance should be considered for WWTPs with high-strength ammonia. On the other hand, the kinetic values of sludge with high-ammonia affinity AOB dominance should be considered for WWTPs with low ammonia.

4. Conclusions

This study shows the link between the ammonia K_s values and the dominant AOB in sludge from full-scale WWTPs and laboratory-scale nitrifying reactors. The dominant AOB species mainly determined the lump ammonia K_s values of the analyzed sludge. This study suggests that different sets of microbial kinetic values for ammonia oxidation may be required for WWTP design and operation to enhance the efficiency and stability of ammonia oxidation.

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