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 (K_s) 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 K_s values for the high-ammonia-level WWTPs and nitrifying reactors were higher than those of the low-ammonia-level plants. Using the K_s values of available AOM cultures as a reference, the K_s 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 WWTPs, 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 values of mixed AOM cultures as a reference, the K_s 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.
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 NH4Cl, NaCl (0.5 g), MgCl2・6H2O (0.4 g), CaCl2・2H2O (0.1 g), KCl (0.5 g), KH2PO4 (0.2 g), H3BO3 (0.03 g), MnCl2・4H2O (0.1 g), CuCl2・6H2O (0.19 g), NiCl2・6H2O (0.024 g), CaCl2・2H2O (0.002 g), ZnSO4・7H2O (0.144 g), Na2MoO4・2H2O (0.036 g), 8 ml of HCl (12.5 M), 1 mL of nonchelated trace element mixture [6], 1 mL of vitamin 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-amoA1F and Arch-amoA2R [7]. The quantification of the AOB amoA genes was performed using the primers amoA1F and amoA2R [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 amoN550D2f and amoN754r [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 [9]. 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 | BOD in influent (mg/L) | BOD in effluent (mg/L) | NH3 in influent (mgN/L) | NH3 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-amoA4F  | 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]        |
| Arch-amoA4R  | 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]        |
| amoA1F       | 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 | [2]        |
| amoA2R       | 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 | [7]        |
| amoN550D2f   | N. oligotropha 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]       |

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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.

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

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 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 modified based on a review by Limpiyakorn et al. [5]. The results showed that the ammonia $K_s$ values of all...
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., $\mu_{max}$, Y, and $k_d$) for ammonia oxidation also differ among AOM groups. For example, the maximum specific growth rate ($\mu_{max}$) of the AOB affiliated with the *Nitrosospira* cluster was higher than that of AOB related to the *Nitrospira* and *Nitroglotropha* 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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