MONITORING THE ABUNDANCE AND

THE ACTIVITY OF AMMONIA-OXIDIZING BACTERIA

IN A FULL-SCALE NITRIFYING ACTIVATED SLUDGE REACTOR

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ABSTRACT

Cell-specific ammonia oxidation rate (AOR) has been suggested to be an indicator of the performance of nitrification reactors and to be used as an operational parameter previously. However, published AOR values change by orders of magnitude and studies investigating full-scale nitrification reactors are limited. Therefore, this study aimed at quantifying ammonia oxidizing bacteria (AOB) and estimating their *in situ* cell-specific ammonia oxidation rates (AOR) in a full-scale activated sludge reactor treating combined

domestic and industrial wastewaters. Results showed that cell-specific AOR changed between 5.30 and 9.89 fmole/cell/hour although no significant variation in AOB cell numbers were obtained ($1.54E+08 \pm 0.22$ cell/ml). However, ammonia removal efficiency varied largely (52-79%) and was proportional to the cell-specific AOR in the reactor. This suggested that the cell-specific AOR might be the factor affecting the biological ammonia removal efficiency of nitrification reactors independent of the AOB number. Further investigation is needed to establish an empirical relationship to use cell-specific AOR as a parameter to operate full-scale nitrification systems more effectively.

INTRODUCTION

Nitrification, the two-step oxidation of ammonia to nitrate, is the key process in the biological nitrogen removal from wastewaters. In the first step, ammonia is oxidized to nitrite by a phylogenetically related group of bacteria called ammonia oxidizers. This step is followed by nitrite oxidation step carried out by nitrite oxidizers. It has been reported that ammonia conversion to nitrite by ammonia-oxidizing bacteria (AOB) is the rate-limiting step in nitrification processes (Grady et al., 1999), therefore high sludge retention time (SRT) is applied to ensure nitrification in wastewater treatment reactors. In aerobic conditions, mainly chemolithoautotrophic *Beta-* and *Gammaproteobacteria* are responsible for ammonia oxidation (Purkhold et al., 2003). Since AOB have very slow growth rate compared to heterotrophs and they are sensitive to inhibitors and changes in environmental conditions (e.g. temperature, pH, SRT), it is essential to better understand the growth kinetics of AOB to enhance process performance and secure effluent discharge limits (Wagner et al., 1995).

Quantification of bacteria in environmental samples is challenging mainly due to the complexity of the samples, low abundance of target bacterial group within the whole community and also due to the methodological limitations. Quantitative PCR and fluorescent *in situ* hybridization (FISH) are the two most common methods used for quantification of bacteria including AOB (Coskuner et al., 2005; Harms et al., 2003). FISH method has specific advantages when the physiological state and the activity of bacteria are important. FISH in combination with activity measurements provides

comprehensive information on the diversity and *in situ* activities of AOB in complex environments such as wastewater treatment reactors. In addition to the number and activity of AOB, cell-specific ammonia oxidation rate (AOR) which is an indicator of the efficiency of a nitrifying bioreactor may serve as a process variable as suggested by Coskuner et al. (2005). Cell-specific AOR is affected by environmental and operational conditions and is different between species. For instance, this value was reported to be between 1.24 to 23 fmol/cell/h for *Nitrosomonas europaea* cultures (Laanbroek and Gerards, 1993) and up to 53 fmol N/cell/hour for AOB isolates (Lydmark, 2006; Belser and Schmidt, 1980). Cell-specific AORs were also estimated in biological reactors and found to be different from those for pure cultures. The values ranged between 0.03 and 49.6 fmol/cell/h (Bellucci et al., 2011; Fujita et al., 2010; Lydmark et al., 2007; Limpiyakorn et al., 2005; Coskuner et al., 2005; Daims et al. 2001). Consequently, it is important to investigate the factors controlling the cell-specific AOR in full-scale systems in order to help engineers to design and operate nitrifying bioreactors more effectively.

This study aims at understanding the relationship between the number of ammonia oxidizers, their cell-specific ammonia oxidation rates and nitrification performance of a full-scale biological reactor treating both industrial and domestic wastewaters during the investigation period.

MATERIALS AND METHODS

Wastewater Treatment Plant

Samples were collected from the biological reactor of a common treatment plant at an

industrial region in Istanbul, Turkey between 2004 and 2006. The treatment plant collects wastewaters from 524 industries which include tannery (163), chemical (130), metallurgical and textile together with domestic wastewaters with a total average daily flow rate of 15000 m^3. The treatment plant is composed of physical and biological treatment units and has the following flow scheme: coarse and fine screening, equalization, primary settling, aerobic biological process and final settling. Biological reactor has a volumetric capacity of 27500 m^3 with 12 m height and 54.8 m diameter.

The characterization of the biological treatment influent was done in a previous study by Cokgor et al. (2008) and given in Supplementary Table 1.

Operational Conditions of the Biological Reactor

The complete-mix activated sludge reactor was operated at various sludge retention times to ensure nitrification under changing ambient temperatures. Dissolved oxygen concentrations and pH values in the biological reactor were kept between 2-4 mg/l and 7.6-7.8, respectively.

Chemical Analysis of the Mixed-Liquor

Chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and ammonia concentrations were measured to monitor the treatment performance for a 19-month period in the plant laboratories. Additionally, mixed-liquor suspended solids (MLSS), mixed-liquor volatile suspended solids (MLVSS) and temperature were measured for the seasonal grab samples. Food-to-microorganisms (F/M) ratio and the sludge retention time

(SRT) were calculated using the volume of the aeration tank and the MLVSS concentrations. Table 1 shows the relevant parameters of the biological reactor for the seasonal samples. All analyses were done according to the Standard Methods (1998).

Sampling

Triplicate grab samples were collected from the mixed-liquor of the activated sludge reactor (aeration tank) at a height of approximately 5 meters in August 2004, December 2004, April 2005, December 2005 and March 2006 to represent the seasonal conditions during the 19-month monitoring period. Samples were transported to the laboratory immediately in a cool-box and fixed for FISH analysis on the same day by keeping in freshly prepared 4% paraformaldehyde fixative solution at $4^{o}C$ for 4 hours and washing with phosphate buffered saline (PBS) solution as described by Amann et al. (1990). Fixed samples were stored at $-20^{o}C$ in 50% ethanol/PBS (v/v) solution until hybridization.

Oligonucleotide Probes

Cyanine labeled probes and competitor oligonucleotides were obtained commercially (Thermo Fischer Scientific, Inc., USA). Six different probes targeting ammonia oxidizing *Betaproteobacteria* (Nso1225, NEU, 6a192) and total *Eubacteria* (Eub338i, Eub338ii, Eub338iii) were used for hybridization. Negative control using NonEub338 probe was also conducted for all samples. NEU probe was used simultaneously with Nso1225 probe since the latter has a single mismatch with *Nitrosococcus mobilis* which is likely to be found in bioreactors treating industrial wastewaters (Juretschko et al., 1998). Table 2 summarizes the probes and the target sites used in the study.

Fluorescent in situ Hybridization and Microscopy

Hybridization protocol according to Manz et al. (1992) was applied for all samples using PTFE covered, 8-well microscopy slides. Hybridization was carried out at $46^{o}C$ for 3 hours using 35% formamide in the buffer. Two washing steps were applied at $48^{o}C$ using 80 μl NaCl concentration. At the end of the washing steps, an anti-fading agent (Citifluor Inc., UK) was applied on slides which were stored at $4^{o}C$ until microscopic examination. Observation of the fluorescent cells was done using Leica TSP2 confocal scanning laser microscope (CSLM) (Leica Microsystems, Heidelberg, Germany). Argon-ion and Helium-neon lasers with excitation wavelengths of 543 nm and 633 nm were used for detection of Cy3 and Cy5 labeled cells, respectively. For each sample, 10 random microscope fields having *z*-series containing 1 to 2 μm optical sections were captured under X600 magnification.

Quantification and the cell specific activity of AOB

Ammonia-oxidizing bacterial numbers were calculated using the method described by Coskuner et al. (2005). This method is based on the mean AOB microcolony volume per unit volume of mixed liquor. Firstly, the number of AOB microcolonies per ml of activated sludge sample was determined using the mean number of AOB microcolonies per field of view of the microscope slide, the area of the sample spot, the area of one field of view and dilution factors used throughout the FISH procedure. Diameters of the AOB microcolonies in each overlapping microscopy images were obtained using DAIME digital image analysis software (Daims et al., 2005) and checked for normality using

Minitab v11 statistical software (Minitab Inc., State College, Pa.). The equation $4/3\,\pi r^3$ was used to calculate the average volume of the AOB microcolonies assuming that they were spherical. The average number of AOB cell per ml of biomass was then calculated using the volume and the number of AOB microcolonies per ml of biomass. Following quantification of AOB cells in the samples, cell-specific ammonia oxidation rates were estimated using the average AOB cell number, the concentration of ammonia converted to nitrite in the activated sludge reactor and the correction factor of 0.9 which takes into account that ammonia is removed by autotrophic nitrification, adsorption and assimilation as described by Daims et al. (2001). The calculated values were expressed as femtomoles of ammonia removed per hour per cell.

RESULTS

Performance of the activated sludge reactor

The investigated activated sludge reactor belongs to a common treatment plant receiving both industrial and domestic wastewaters. COD removal efficiency changed between 84-91% (Table 3) whilst TKN and ammonia removal efficiencies were between 51-80% and 46-85%, respectively (Figure 1). During the 19-month monitoring period, the highest TKN and ammonia removal efficiencies were observed in July 2005 (80.2 and 85.3%, respectively) whereas the lowest removal efficiencies were in November 2005 (51.3 and 46.5%, respectively). However, among the sampling months for AOB quantification, the highest ammonia removal efficiency was observed in August 2004 which had the highest ambient temperature (24 ± 0.8^{o}C) and 10 days of sludge retention time; whereas the lowest was observed in April 2005 although it had moderate temperature and sludge

retention time ($14 \pm 0.6^{\circ}$C and 12 d).

AOB cell numbers and cell-specific ammonia oxidation rates

Ammonia-oxidizing bacteria in the activated sludge samples were quantified using oligonucleotides specific to all known ammonia oxidizing *Betaproteobacteria* together with *Nitrosomonas oligotropha* lineage. Eubacterial probes were also used to detect most of eubacterial community; using these probes with AOB probes simultaneously increased the signal intensity and the reliability of detected colonies, therefore, overlapping images of the two probe sets were used for quantification (Supplementary Figure 1). The results revealed that AOB cell numbers had a mean value of 1.54E+08 $\pm$ 0.22 cell/ml and did not exhibit a significant change in the analyzed samples (ANOVA, p=0.532; Figure 2). Cell-specific AOR varied between 5.30 and 9.89 fmole/cell/hour (Table 4) and it was proportional to the ammonia-removal efficiency (Figure 3). No direct relationship was established between the AOB abundance, influent TKN or ammonia concentrations with the cell-specific ammonia oxidation rates (ANOVA, p>0.5).

DISCUSSION

The results revealed that AOB cell numbers (1.54E+08 $\pm$ 0.22 cell/ml) did not change significantly although ammonia removal efficiency varied broadly in the analyzed samples. Cell-specific AOR which varied between 5.30 and 9.89 fmole/cell/hour. In comparison to previously reported values for pure AOB cultures (0.03-53 fmol N/cell/hour) and for full-scale activated sludge plants (0.03 and 49.6 fmol/cell/h), the values that were found in this study did not change in a wide range and also were lower.

This suggests a relatively stable operation of the nitrification reactor as high cell-specific AOR values might be obtained for reactors close to failure (Coskuner et al., 2005). The proportional variation between the cell-specific AOR and ammonia removal efficiency together with the insignificant change in AOB abundance suggested that the cell-specific AOR might be the factor affecting the nitrification efficiency independent of the AOB abundance. This result contradicts the study by Coskuner et al. (2005) who suggested that the variation in cell-specific AOR is due to the variation in the number of AOB in domestic wastewater treatment reactors. On the other hand, our results might be attributed to low ammonia removal efficiencies occurred within the reactor (52.7%-79.6%) as suggested by Bellucci et al. (2011) who found a linear relationship between the cell-specific AOR and the AOB abundance when only ammonia removal was higher than 95% in lab-scale nitrifying bioreactors. Moreover, the lack of correlation between the number of AOB and their cell-specific activities might be explained by the change in AOB composition during the investigation period. Population shift might have occurred within AOB lineages which kept the total AOB number almost constant whilst the cell-specific activities varied. Since FISH probes used in this study targets all known betaproteobacterial AOB collectively, it is not known if the system contained a similar AOB diversity during the study. Over a long operational period, mixed industrial wastewater components might have led different AOB community structure in the biological reactor. As stated by Daims et al. (2001) and Rowan et al. (2003), a plant with greater diversity can adapt to changes in environmental conditions and obtain more stable efficiency. This might be the case in our reactor of interest which might have had different predominant AOB taxa to overcome the changes in operational and

environmental conditions whilst low AOB diversity might have caused low ammonia removal efficiencies observed in April and May 2005. Other molecular techniques (such as DGGE and sequence analysis) may help obtain detailed bacterial diversity analysis over longer periods.

Evaluation of the F/M ratio and the cell-specific AOR implied that the change in the cell-specific AOR might be driven by the change in F/M ratio. Although the relationship was not linear between the two parameters, it is plausible to suggest that the amount of influent carbon together with SRT might play a role on the metabolism of AOB.

One should bear in mind that it is a challenging task to precisely evaluate the effects of environmental and operational conditions on cell-specific AOR in full-scale bioreactors as these systems might be affected by a combination of factors over time such as sudden changes in influent wastewater characteristics. In our study, five samples to represent different seasons over a 19-month period were collected. More frequent sampling would help relate the changes in bacterial abundance and activity to environmental and operational conditions of the treatment systems. It should also be noted that the estimated AOB numbers might have been biased as the probe set used in this study has mismatches with some AOB species and not 100% sensitive (Purkhold et al., 2000). Furthermore, the FISH probes used in this study target all described AOB only within *Betaproteobacteria*; however, the biological reactor of interest might have contained other AOB within *Gammaproteobacteria* such as *Nitrosococcus halophilus*, *Nitrosococcus oceani* and *Nitrosococcus sp*. (Purkhold et al., 2000). In addition, there is a possibility that ammonia-

oxidizing archaea existed in the low-oxygen microhabitats within the bioreactor which would have caused the overestimation of the ammonia removal efficiency by AOB (Park et al., 2006).

Despite the limitations of the quantification technique used and possible errors caused by the estimation methods of the AOB abundance and activity, the cell-specific ammonia oxidation rate could be considered as an operational parameter. Coskuner et al. (2005) used the same principle to calculate the AOB numbers and estimate cell-specific AOR and they quantified more than 95% of detectable AOB. They also suggested that there is a threshold cell-specific AOR below which stable nitrification performance might be expected. Operating a treatment reactor with low AOB numbers but high activity within a certain limit may help reduce the aeration costs whilst avoiding the failure of nitrification systems.

CONCLUSION

This study is of importance as it provides information on the treatment performance of full-scale activated sludge systems, AOB abundance and cell-specific activity. This study shows that cell-specific ammonia oxidation rate may not be directly related to influent amount of nitrogen, sludge retention time or the AOB numbers in full-scale treatment plants but may be related to F/M ratio and may affect the nitrification performance of the reactor. Therefore, further investigation needs to be conducted to establish an empirical relationship if cell-specific AOR is to be used as an operational parameter for full-scale nitrification systems.

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