Ammonium-oxidizing bacteria facilitate aerobic degradation of sulfanilic acid in activated sludge

Gang Chen, Maneesha P. Ginige, Anna H. Kaksonen and Ka Yu Cheng

ABSTRACT

Sulfanilic acid (SA) is a toxic sulfonated aromatic amine commonly found in anaerobically treated azo dye contaminated effluents. Aerobic acclimatization of SA-degrading mixed microbial culture could lead to co-enrichment of ammonium-oxidizing bacteria (AOB) because of the concomitant release of ammonium from SA oxidation. To what extent the co-enriched AOB would affect SA oxidation at various ammonium concentrations was unclear. Here, a series of batch kinetic experiments were conducted to evaluate the effect of AOB on aerobic SA degradation in an acclimatized activated sludge culture capable of oxidizing SA and ammonium simultaneously. To account for the effect of AOB on SA degradation, allylthiourea was used to inhibit AOB activity in the culture. The results indicated that specific SA degradation rate of the mixed culture was negatively correlated with the initial ammonium concentration (0–93 mM, $R^2 = 0.99$). The presence of AOB accelerated SA degradation by reducing the inhibitory effect of ammonium ($\geq$10 mM). The Haldane substrate inhibition model was used to correlate substrate concentration (SA and ammonium) and oxygen uptake rate. This study revealed, for the first time, that AOB could facilitate SA degradation at high concentration of ammonium ($\geq$10 mM) in an enriched activated sludge culture.

Key words | 4-aminobenzenesulfonic acid, allylthiourea, azo dye, Haldane model, sulfonated aromatic amine

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INTRODUCTION

Sulfanilic acid (SA) (also known as 4-aminobenzensulfonic acid) is among the group of sulfonated aromatic amine compounds widely used as an intermediate for the synthesis of chemical compounds such as sulfonated azo dyes, pesticides and pharmaceuticals (Tan *et al.* 2005). The main resource of SA is from the reductive decolorization of sulfonated azo dyes (O'Neill *et al.* 1999). Due to its negatively charged sulfonyl group, SA is highly water soluble and has been found to be even more toxic and carcinogenic than its precursors (Chung & Cerniglia 1992; Oh *et al.* 1997; O'Neill *et al.* 1999). In addition, SA can also result in environmental and health problems (Chung & Cerniglia 1992; Oh *et al.* 1997). Therefore, SA-contaminated wastewaters should be treated before discharging to the environment.

Aerobic microbial oxidation of SA using pure culture and co-culture has been considered as a viable option to treat SA-contaminated effluent (Singh *et al.* 2006; Wang *et al.* 2009; Gan *et al.* 2011; Chen *et al.* 2012). Since SA consists of an amino group, upon microbial oxidation an ammonium ion is also released into the bulk (reaction (1)). In a mixed microbial system such as activated sludge, this released ammonium could potentially serve as a substrate for nitrifying bacteria to form nitrite and/or nitrate via nitrification (Davies *et al.* 2006).

$$C_6H_7NSO_3 + 7O_2 \rightarrow NH_4^+ + SO_4^{2-} + 6CO_2 + H_2O + H^+ \quad (1)$$

Nitrification is a well-known two-step oxygen-consuming process, in which ammonium is first oxidized into nitrite by ammonium-oxidizing bacteria (AOB) (reaction (2)), and the nitrite is subsequently oxidized into nitrate by nitrite-oxidizing bacteria (NOB) (reaction (3)) (Carrera *et al.* 2004).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (2)$$

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$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (3)$$

$$NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O \quad (4)$$

In a mixed microbial culture specifically enriched for SA degradation, nitrifiers (AOB and NOB) may compete with the SA-oxidizing bacteria (hereafter termed as SAB) for dissolved oxygen (DO) and as such the overall SA degradation kinetics may be hampered (Chen *et al.* 2012). However, it is also possible that when DO is not limiting the co-existence of AOB would facilitate SAB to oxidize SA via a hypothetical relationship between SAB and AOB (Figure 1). Since ammonium is the end-product of SA oxidation (reaction (1)), elevated concentrations of ammonium may suppress SA oxidation due to product inhibition. The removal of ammonium by AOB (via nitrification) may alleviate such inhibition and hence facilitate the SAB to oxidize SA. This hypothesis has not been validated yet. Hence, the objective of this study was to verify the relationship between SAB and AOB in a SA-acclimatized activated sludge at various ammonium concentrations. To elucidate the effect of AOB on SA oxidation at various ammonium concentrations, AOB activity in the mixed culture was selectively suppressed by using allylthiourea (ATU), a well-known specific nitrification inhibitor (Silva *et al.* 2009). Substrates' (SA and ammonium) oxidation and oxygen uptake kinetics of the culture were evaluated in a series of batch experiments. Understanding the role of AOB in aerobic degradation of SA would aid process optimization of aerobic treatment of SA-contaminated wastewater.

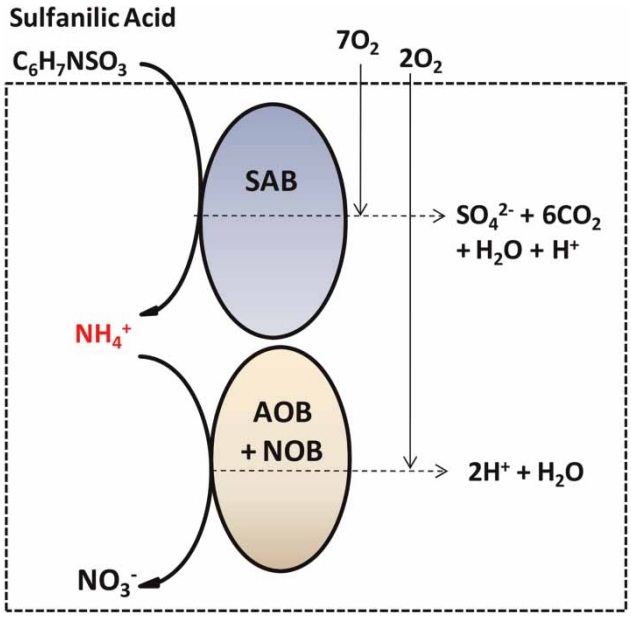

Figure 1 | Hypothetical symbiotic relationship between SA-oxidizing bacteria (SAB) and AOB in a mixed microbial culture with SA as a sole carbon and energy source and oxygen as a primary electron acceptor. NOB = nitrite oxidizing bacteria.

METHODS

Chemicals

SA (4-aminobenzene sulfonic acid, CSC number: 121-57-3) and ATU (CSC number: 109-57-9) were purchased from Sigma-Aldrich (Australia). The working stock solution of SA (5,000 mg/L) and ATU (3,000 mg/L) were prepared by dissolving the chemical in deionized water. Working concentrations of SA and ATU in different experimental runs were prepared by adding a predetermined volume of the stock solution to the medium. All other chemicals used in this study were of analytical grade.

Inoculum and culture medium composition

Activated sludge was obtained from a municipal wastewater treatment plant in Perth, Australia, and was stored at 4 °C prior to use. The medium used in the experiment consisted of (mg/L): $NaHCO_3$ 125, $MgSO_4 \cdot 7H_2O$ 51, $CaCl_2 \cdot 2H_2O$ 300, NH_4Cl 125, $FeSO_4 \cdot 7H_2O$ 6.25, and 1.25 ml/L of trace element solution, which contained (g/L): ethylenediamine tetraacetic acid 15, $ZnSO_4 \cdot 7H_2O$ 0.43, $CoCl_2 \cdot 6H_2O$ 0.24, $MnCl_2 \cdot 4H_2O$ 0.99, $CuSO_4 \cdot 5H_2O$ 0.25, $NaMoSO_4 \cdot 2H_2O$ 0.22, $NiCl_2 \cdot 6H_2O$ 0.19, $NaSeO_4 \cdot 10H_2O$ 0.21, H_3BO_4 0.014, and $NaWO_4 \cdot 2H_2O$ 0.05 (Chen *et al.* 2012). The medium was adjusted to pH 7.0 with phosphate buffer (K_2HPO_4 and KH_2PO_4, final concentration 15–30 mM).

Bioreactor of SA degradation

A 2 L continuously stirred tank reactor with a working volume of 1.5 L was used throughout the study. The SA-enriched activated sludge used in this study was pre-acclimated using SA as the sole source of carbon and energy for over 180 days (SA concentration of 500–1,000 mg/L). The reactor was operated in batch mode at room temperature. The suspension liquor was continuously stirred at about 300 rpm using an overhanging turbine impeller stirrer to maximize mass transfer. Aeration was provided to the culture at specified flow rates (1.74 L/min). A DO sensor and

process monitor (TPS Pty Ltd, Australia) were used to measure the DO concentration in the suspension liquor. The DO data were periodically recorded into an Excel spreadsheet using a LabVIEW™ computer program.

Experimental procedures

Capacity of the enriched activated sludge for SA and ammonium oxidation

Batch experiments were performed to evaluate the kinetics of SA and ammonium oxidation by the enriched activated sludge. Changes in SA concentration, ammonium concentration and chemical oxygen demand (COD) were measured over time. SA degradation products such as nitrite, nitrate and sulfate were also quantified. The initial SA concentration was 3.3 mM, the mixed liquor suspended solids (MLSS) concentration in the culture was maintained at approximately 58,500 mg/L and the aeration rate was controlled at 1.74 L/min. All experimental settings were evaluated more than once to ensure reproducibility of the chemical profile trends for meaningful comparison. In this paper, only the representative profiles were included to demonstrate the biological process that took place in the reactor.

SA degradation of the enriched culture at different initial ammonium concentrations

To evaluate the effect of ammonium on SA oxidation, a series of batch experiments were conducted to obtain SA degradation rates at different initial ammonium concentrations (0–93 mM). The initial SA concentration in these experiments was maintained at 3–3.2 mM. This SA concentration range was chosen because it allowed the maximum specific SA degradation rate and oxygen consumption rate of the established SA-degrading culture (Chen *et al.* 2012). The MLSS concentration of the enrichment was maintained at 5,500 mg/L consistent with our previous work. The aeration rate was controlled at 1.74 L/min to supply enough oxygen in the experiments (Chen *et al.* 2012). Changes in SA concentration over time at different initial ammonium concentrations were compared, and used to derive the specific SA degradation rates.

SA degradation by ATU treated cultures

To determine whether the co-enriched AOB were involved in SA biodegradation, ATU (0.2 mM) was added to the mixed culture to selectively inhibit the nitrification activity of AOB (Silva *et al.* 2009). A series of batch experiments with and without ATU amendment were carried out to evaluate the effect of AOB on SA degradation. In these runs, the initial SA concentration was 3–3.2 mM, ammonium concentration was 10 mM, MLSS concentration was 5,500 mg/L and the aeration rate was 1.74 L/min. Changes in SA concentration over time in the presence and absence of ATU were compared, and ammonium, nitrite, nitrate and sulfate were measured.

Oxygen uptake kinetics of SAB and AOB

A series of batch experiments were conducted with various initial SA or ammonium concentrations to determine the respective oxygen uptake kinetics of SAB and AOB. The Haldane competitive substrate inhibition kinetics model (Equation (5)) (Han & Levenspiel 1988) was used to obtain kinetic parameters for oxygen uptake rate (OUR) by SAB and AOB in separate runs.

$$U = U_{\max}\frac{C_s}{K_s + C_s + \left(C_s^2/K_I\right)} \tag{5}$$

where U is specific OUR (mg O_2/(g MLSS · h)), $U_{\max}$ is maximum specific OUR (mg O_2/(g MLSS · h)), C_s is initial substrate concentration (mg/L), K_s is half-saturation constant (mg O_2/L) and K_I is inhibition constant (mg/L).

The dissolved OUR was determined using the data obtained from the DO sensor. At predefined time points, 50 mL of the mixed liquor sample were taken from the 2 L bioreactor and immediately transferred to a separate, 50 mL glass vessel equipped with both a magnetic stirrer and DO sensor. The liquor was pre-aerated with moist air until DO became steady. Thereafter, the aeration was terminated and the OUR was derived from the change in DO concentration over time. The Haldane model was used to correlate the relationship between OUR and the initial concentration of SA and ammonium, respectively.

Analytical methods

All samples taken from the bioreactor were immediately filtered through a 0.2 μm sterile filter (0.8/0.2 μm Supor® membrane, Pall Life Sciences) to remove biomass prior to chemical analysis. SA concentration of the filtrate was determined using a UV-spectrophotometer (Cary 50, Varian) at 250 nm (Chen *et al.* 2012). The samples were diluted to obtain a SA concentration between 0 and 25 mg/L, which

gave a linear standard curve with the absorbance ($R^2 > 0.999$). COD was measured with a closed reflux dichromate COD method (Hach Method 8000, Hach Ltd). SO_4^{2-}, NH_4^+-N, NO_3^--N and NO_2^--N were analysed by ion chromatography (ICS-3000, Dionex). MLSS was measured according to *Standard Methods* (APHA 1995).

RESULTS AND DISCUSSION

Co-enrichment of AOB in an enriched activated sludge culture using SA as the sole carbon and energy source

The concomitant release of ammonium from the microbial SA oxidation was expected to facilitate co-enrichment of AOB in the mixed aerobic culture. To validate whether AOB was co-enriched in the enriched SA-degrading culture, the major metabolic ionic species (i.e. ammonium, nitrite, nitrate and sulfate) were quantified over the course of SA degradation (Figure 2). Clearly, the enriched mixed culture illustrated the capacity for SA degradation and mineralization. Complete degradation of SA (3.3 mM) with a high COD removal rate (97.1%) was achieved after around 7.5 h, accompanied with a near stoichiometric release of sulfate (3.2 mM SO_4^{2-} released from 3.3 mM SA) (reaction (1)) (Chen *et al.* 2012). In contrast to other pure- and co-culture studies (in the absence of AOB) where ammonium released from SA mineralization accumulated in the system (Singh *et al.* 2006; Wang *et al.* 2009; Gan *et al.* 2011), our mixed culture system showed concomitant accumulation of both nitrite (0.2 mM) and nitrate (3.6 mM), revealing the co-enrichment of AOB in the mixed culture.

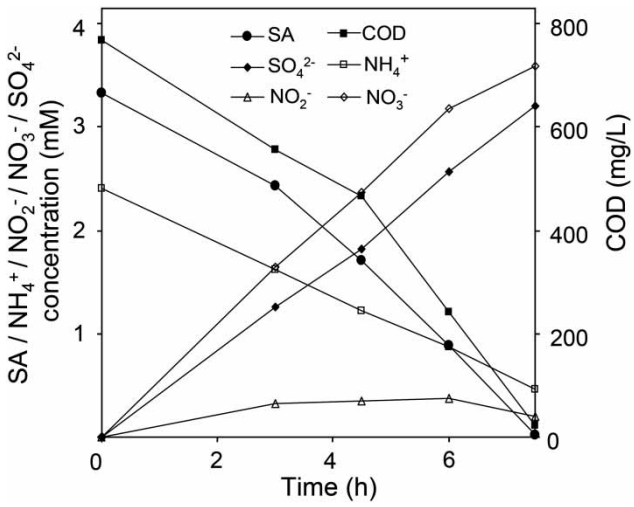

Figure 2 | Concentrations of SA, COD, sulfate, nitrate and nitrite during the removal of SA (3.3 mM) and ammonium by the enriched activated sludge (MLSS = 5,800 mg/L, aeration rate = 1.74 L/min).

High concentration of ammonium-suppressed SA degradation by the enriched culture

The effect of ammonium concentration on SA degradation in the enriched culture was investigated (Figure 3). When ammonium was not provided, the enriched culture only required about 5.5 h to completely degrade the SA at a specific SA degradation rate of 0.14 mM/(g MLSS · h) (Figure 3). When the initial ammonium concentrations increased to 10 and 37 mM, the time required for the complete SA degradation increased to 5.7 and 6.3 h, respectively, while the specific SA degradation rate decreased from 0.14 to 0.13 and 0.11 mM/(g MLSS · h), respectively. The differences in the range from 0 to 37 mM ammonium

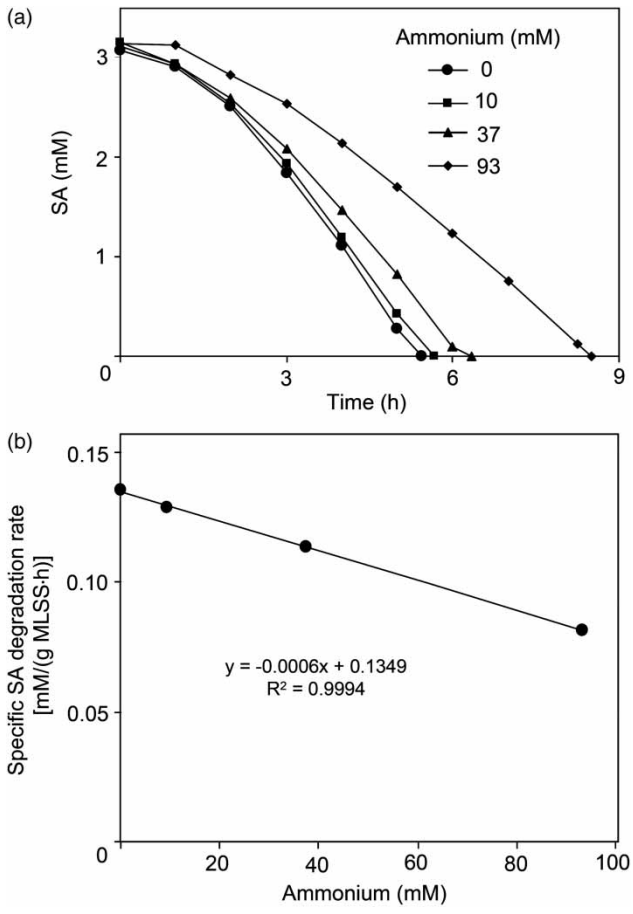

Figure 3 | (a) Effect of initial concentration of ammonium on SA degradation. (b) Relationship between specific SA degradation rate and initial ammonium concentration (SA = 3.1–3.2 mM, MLSS = 5,500 mg/L and aeration rate = 1.74 L/min).

for SA degradation were not obvious, which may be due to the co-existence of co-enriched AOB in the mixed culture.

A clear inhibition of SA degradation was observed when the initial ammonium concentration increased to 93 mM. This resulted in a lag period of about 1 h and an increased time (8.5 h) required for complete SA degradation, with the specific SA degradation rate remarkably reduced to 0.08 mM/(g MLSS · h) (Figure 3(a)). Overall, these results indicate that SA degradation by the enriched mixed culture was negatively affected at high concentrations of ammonium (Figure 3(b)).

Co-existence of AOB could reduce the inhibitory effect of ammonium on SA degradation by the SAB in the mixed culture

The co-enriched AOB in the mixed culture was expected to facilitate SA degradation by reducing ammonium inhibition. To test whether AOB would facilitate SA degradation by the enriched culture, a specific nitrification inhibitor (here ATU) was added to the mixed culture to selectively suppress the AOB activity (Figure 4). The addition of ATU did not affect SA degradation in the absence of ammonium, suggesting that the SA-degrading capacity of the enriched SAB was not inhibited by ATU (Figure 4(a)). When AOB activity was not suppressed (i.e. no ATU addition), the presence of ammonium (10 mM) only slightly reduced the specific SA oxidation rate by 7.1% (0.14 to 0.13 mM/(g MLSS · h)). However, 23.1% reduction (0.13 to 0.10 mM/(g MLSS · h)) in specific SA oxidation rate was noted in the presence of ammonium when AOB activity was suppressed by the ATU (Figure 4(a)). The result indicated that the AOB activity could sustain efficient SA degradation in the mixed culture when ammonium initially existed at high level (10 mM). The positive effect of AOB in facilitating SA degradation could be further substantiated by the release of sulfate (a product of SA oxidation) to the bulk (Figure 4(b)). Clearly, a remarkably slower release of sulfate was recorded when the AOB activity was inhibited, indicating a positive effect of AOB on SA degradation (Figure 4(b)).

The presence of AOB in the culture had continuously converted the ammonium into nitrate while an increasing ammonium concentration in the culture was noted when AOB activity was suppressed (Figure 4(b)). In the absence of ATU, ammonium concentration decreased from 10 to 8.76 mM and nitrate increased to 2.83 mM over the duration of SA degradation (Figure 4(b)). In contrast, in the presence of ATU, the ammonium concentration increased from 10 to 11.76 mM and no nitrate was produced, indicating the

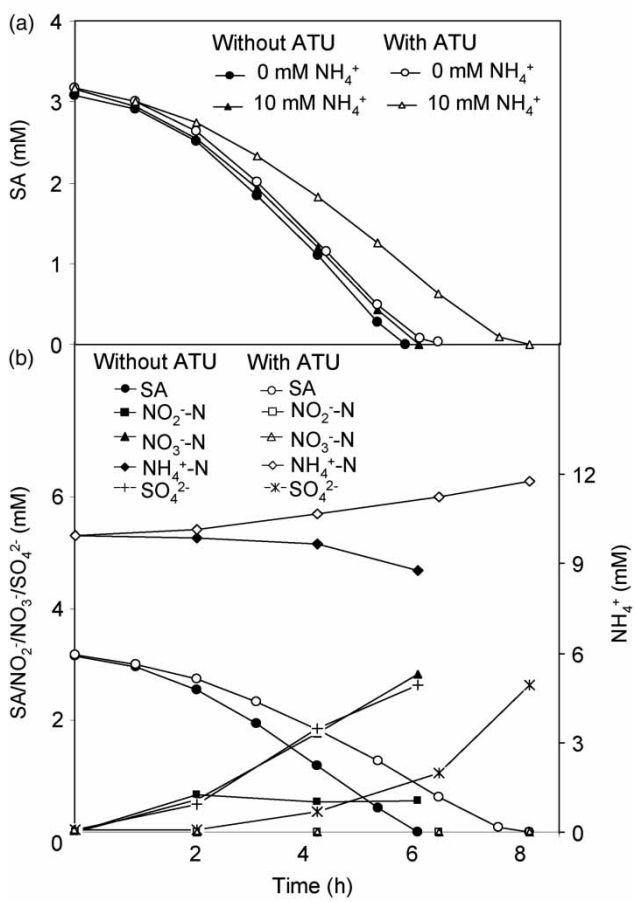

Figure 4 | (a) SA degradation with and without addition of ATU (0.2 mM, used as nitrification inhibitor). (b) Production of sulfate, ammonium, nitrite and nitrate with and without addition of ATU in the presence of ammonium with initial concentration of 10 mM. (SA = 3.2 mM, MLSS = 5,500 mg/L and aeration rate = 1.74 L/min.)

release of ammonium from SA oxidation catalysed by the SAB and the lack of AOB activity (Figure 4(b)). These results suggest that the co-existence of AOB could reduce the inhibitory effect of ammonium on SA degradation by the SAB in the mixed culture.

Oxygen uptake kinetics of SAB and AOB

Since DO is the common electron acceptor for both SAB and AOB, quantifying the oxygen uptake kinetics of these two groups of bacteria may help understand the competition between SAB and AOB for DO in the system. Figure 5 illustrates the relationship between specific OUR and the initial concentration of SA and ammonium, respectively. The kinetic data were fitted into a Haldane model (Equation (5)) to derive the respective half-saturation constant (K_s), maximal specific OUR (U_{max}) and substrate inhibition constant

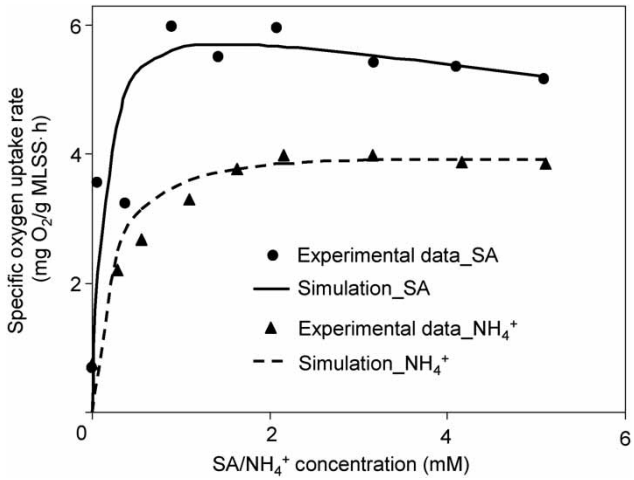

Figure 5 | The relationship between specific oxygen uptake rate and initial concentration of SA or ammonium.

Table 1 | Results of the Haldane model parameters for the specific OUR data of SAB and AOB

Parameters	SAB	AOB
U_{max} [mg O_2/(g·h)]	6.51	4.32
K_s [mM]	0.11	0.20
K_I [mM]	22.1	82.8

K_s = half-saturation constant (SA for SAB; ammonium for AOB); U_{max} = maximal specific OUR; K_I = substrate inhibition constant (SA for SAB; ammonium for AOB).

(K_I) (Table 1). The K_s value for SAB was almost 2-fold lower than that for AOB, whereas the U_{max} value for SAB was 1.5-fold higher than that of AOB. These indicate that SAB appear to be more capable of competing for oxygen with AOB for substrate oxidation. This observation is reasonable given that the mixed culture was acclimatized using SA as the sole carbon and energy source. Hence, the co-enrichment of AOB in the culture depended exclusively on the activity of SAB, which released the substrate (ammonium) for the AOB using oxygen as electron acceptor (Figure 1). A higher affinity of SAB towards oxygen may ensure effective metabolic activities for both SAB and AOB in the mixed system. Nevertheless, future studies are warranted to elucidate in detail the metabolic relationship between SAB and AOB in the enriched culture.

Implication of the finding

Numerous studies have investigated the aerobic degradation of sulfonated amines using pure microbial culture or co-cultures (e.g. Perei *et al.* 2001; Singh *et al.* 2004; Wang *et al.* 2009; Gan *et al.* 2011). However, using pure cultures or co-cultures for wastewater treatment is practically difficult, and mixed microbial systems such as activated sludge are preferred. Our previous study had demonstrated the effectiveness of using activated sludge to achieve complete SA mineralization (Chen *et al.* 2012). Interestingly, the release of ammonium from SA degradation resulted in a co-enrichment of AOB and thus a build-up of nitrate. This observation was in contrast to the above mentioned pure- and co-cultures studies where ammonium accumulation was found to be predominant due to the lack of AOB. Undoubtedly, understanding the role of such 'SA-dependent' AOB in the activated sludge process is crucial for process optimization.

In the present study, the effects of AOB on SA oxidation and the kinetics of oxygen consumption of SAB and AOB in a mixed aerobic culture were elucidated for the first time. Although both AOB and SAB require oxygen as a common electron acceptor, the findings clearly showed that at elevated ammonium concentrations, SA degradation was facilitated by AOB. This implies that AOB activity (i.e. ammonia oxidation) should be conserved and/or optimized to ensure stable and efficient SA removal during the wastewater treatment process.

CONCLUSIONS

Release of ammonium during aerobic SA biodegradation resulted in a co-enrichment of AOB in an activated sludge culture. This is the first study to examine the role of AOB in a mixed microbial system for SA degradation. The results obtained verify that the AOB could facilitate SA degradation at high concentration of ammonium in the SA-acclimatized activated sludge culture. The following points are highlighted from this work.

- AOB were co-enriched in an activated sludge using SA as the sole carbon and energy source.
- The presence of AOB in the enriched activated sludge had a positive effect on SA degradation at an elevated ammonium concentration (here 10 mM).
- DO was consumed more efficiently by SAB than AOB in the mixed culture.

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