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ORIGINAL ARTICLE

Activity enhancement of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in activated sludge process: metabolite reduction and CO_2 mitigation intensification process

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Abstract

The interaction of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) is of considerable importance in nitrification process. Ecophysiological interactions between the communities of AOB and NOB were investigated by monitoring NO_2^- as the intermediate compound in an organic carbon-depleted nitrifying activated sludge fed only NH_4^+ as a nitrogen source (40 mg/L). The presence of boom and bust (feast and famine) cycle successfully indicates the activity cycles of AOB and NOB through cultivation-dependent method. The maximum growth rate and yield for AOB in nitritation-dominant period were (0.67 day^{-1}, 0.17 gVSS gN^{-1}) and for NOB in nitratation-dominant period were (0.71 day^{-1}, 0.072 gVSS gN^{-1}). Soluble microbial products (SMP) and extracellular polymeric substances (EPS) generated by AOB were 1.2 and 1.8 mg/L, respectively, while NOB produced 0.6 mg/L of SMP and 1 mg/L of EPS. While NOB were low in utilization-associated products (UAP) (0.07 mg/L) and biomass-associated products (BAP) (0.12 mg/L), AOB were higher in UAP (0.15 mg/L) and BAP (0.3 mg/L). The continuation presence of zero C/N ratio, in either inlet ratio or net available ratio for the microbial community, can prolong and enhance nitratation process. NOB enrichment and nitratation intensification strategy through zero C/N ratio are able to reduce remarkably microbial metabolites 50% lower than conventional process and enhance nitrification efficiency in activated sludge-involved processes.

Keywords Activated sludge · Nitrite · AOB · Fouling · NOB · Nitrification · SMP · EPS

Abbreviations

AOB	Ammonium oxidizing bacteria
BAP	Biomass-associated products
CAS	Conventional enriched activated sludge
C/N	Organic carbon to nitrogen loading rate
COD	Chemical oxygen demand
CTR_{meas}	Measured carbon dioxide uptake rate
DO	Dissolved oxygen
EPS	Exteracellular polymeric substance
HPR_{meas}	Hydrogen ion production rate
NAS	Nitrifier enriched activated sludge
NOB	Nitrite-oxidizing bacteria
r_{NH_3}	Rate of ammonium consumption
$r_{NO_2^-}$	Rate of nitrite accumulation
$r_{NO_3^-}$	Rate of nitrate production
r_{O_2}	Rate of oxygen consumption
SMP	Soluble microbial products
TOGA	Titrimetric and offgas analyzer
UAP	Utilization-associated products
Y_A	Yield (subscript 1 = nitritation; subscript 2 = nitratation)
μ_{Amax}	Maximum growth rate

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Introduction

Biological treatment is widely used for municipal and industrial wastewaters (Massot et al. 2012). The microbial metabolites produced during biological treatment in the forms of soluble microbial products (SMP) and extracellular

polymeric substances (EPS) are mainly composed of proteins, carbohydrates, and humic substances (Kunacheva et al. 2017). These metabolites create a residual organic matter in secondary effluent (Zhang et al. 2017). Due to their impacts on the physio-chemical properties of microbial aggregates in the sludge, SMP are considered as major sludge floc component leading to keep the floc in a matrix. Conversely, these major components are viewed as the key foulant causing irreversible biofouling of membrane bioreactors. During the post-treatment process, SMP and EPS are regarded as precursors for disinfection by-products, which aggravates the treatment process (Krasner et al. 2009; Shariati et al. 2013). Therefore, microbial metabolites are undergoing a revolution in terms of research focus in the field of environmental biotechnology.

Previous studies (Xie et al. 2012; Dolinšek et al. 2013) focused on unified theory which explains the growth of heterotrophic bacteria supported by nitrifiers as primary producers. The theory indicates the supporting microbial growth mechanism via the give-and-take between autotrophs and heterotrophic bacteria through the production of utilization-associated products (UAP), biomass-associated products (BAP) by autotrophs and carbon dioxide production by heterotrophs. Figure 1 shows the summary of unified theory which points out autotrophic and heterotrophic collaboration for assimilation (Ni et al. 2011). On the contrary, competition between heterotrophs and nitrifiers for terminal electron acceptor O_2 and the production rate of total biomass not only establish a complicated interdependent microbial network but also can influence on SMP and EPS generation (Hunt et al. 2018). Relative population of autotrophs and heterotrophs depends heavily on C/N ratio available to microbial community. Under different operational conditions such as HRT, SRT, DO concentration, temperature, and substrate concentration the responses of autotrophic nitrifiers are divergent, which in turn influence metabolite production (Hu et al. 2003). In low C/N ratios, nitrifiers dominate heterotrophs and enhance the nitrification efficiency (Ma et al. 2013). Ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), contributors of two phylogenetically unassociated congregations of lithoautotrophic bacteria, perform nitrification process via a symbiosis interaction. The limiting step in nitrification process generally is attributed to the nitritation process (Carrera et al. 2003; Su 2012; Raimonet et al. 2015; Silva et al. 2018; Zeng et al. 2018). Besides the thermodynamic limitation and competition of heterotrophs with nitrifier for oxygen and space, AOB compete with NOB for oxygen and alkalinity. As a result, elimination of external organic carbon by adjusting C/N ratio to zero can resolve the inhibitory role of heterotrophs in nitrification (Ma et al. 2013).

Dissecting nutrient flow through AOB to NOB is a non-trivial task which needs to detect the uptake and assimilation of substrates such as NH_4^+ and NO_2^- (Kindaichi et al. 2004). To optimize nitrification efficiency, the growth balance between AOB and NOB become a vital factor especially

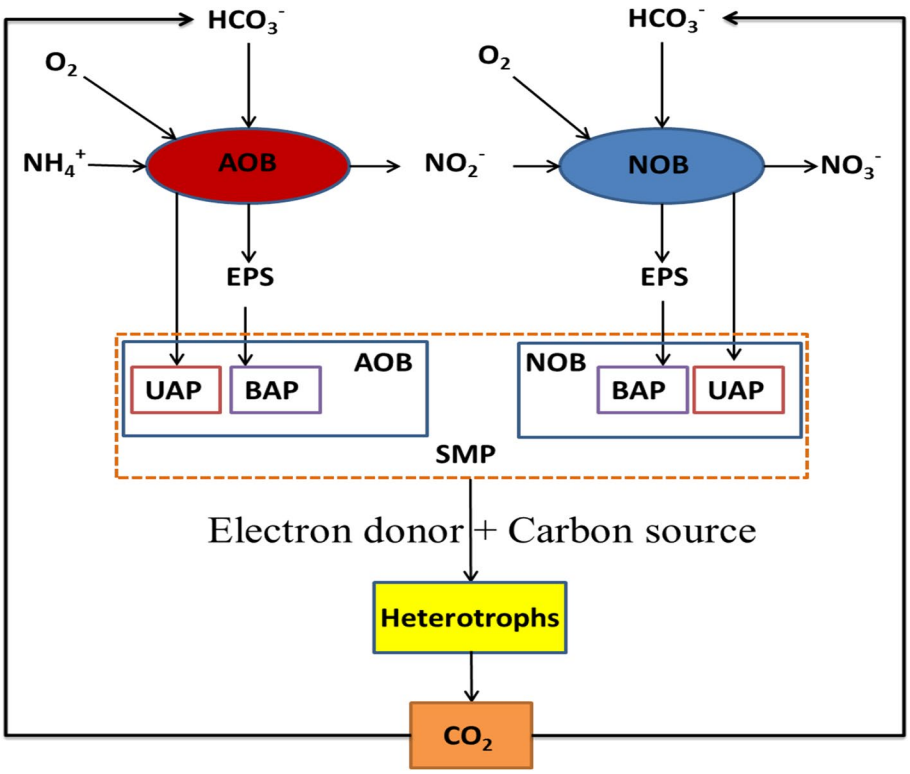

Fig. 1 Interaction of AOB, NOB, and heterotrophs according to the unified theory

when the advanced water treatment process is used (Yao and Peng 2017). This balance can be adjusted through regulation of nitratation process.

It would be also beneficial to analyze the distribution of metabolites in AOB and NOB since the pattern of these distributions can provide significant hints about the nature and specificity of complicated interactions between AOB and NOB in flocs as well as biofilms. Metabolite distribution in nitrification can be highly significant especially in N-riched advanced wastewater treatment processes. A neglected area in the field of autotrophic nitrification led to the question of how specific such complicated interactions might be employed in order to minimize EPS and SMP without compromising assimilation.

In the present study, we have developed an innovative solution to specifically investigate two theories: (1) growth detection of AOB and NOB by cross-feeding of NOB by AOB through a viable non-molecular analysis; (2) quantification of different microbial metabolites by AOB and NOB based on unified theory in the N-riched media.

Materials and methods

Nitrifiers enrichment through particular feeding method

Enrichment of nitrifiers was conducted using the inorganic feed to activated sludge, which was obtained from local municipal wastewater treatment plant during 75 days. In order to monitor the growth of nitrifiers, performances of nitrifier-enriched activated sludge (NAS) and conventional activated sludge (CAS) were compared in terms of nitrification efficiency and COD removal in the batch mode. Nitrification efficiency in NAS and CAS was 100% vs. 43%, while the COD removal in NAS and CAS was 9% vs. 65%. Detecting different parameters such as alkalinity, pH, and nitrate also fortified the enrichment of nitrifiers. More data regarding the enrichment process are found in the related paper (Sepehri and Sarrafzadeh 2018).

Process description and bioreactor set-up

In order to monitor and distinguish the growth of AOB and NOB, a Plexiglas airlift reactor with a working volume of 20 L was selected. The internal diameter was 15 cm, and height of the reactor was 100 cm. In order to measure energy solely through chemical oxidation, the reactor was opaque in color. It is significant to know that the bioreactor was equipped with a thermostatic jacket and temperature was maintained at 30 ± 0.5 °C. Air was supplied through an air diffuser connected to an air pump. Aeration density was monitored and controlled via an airflow meter to keep DO concentration at 3–4 mg/L. For the sake of keeping SRT at 7 days, the extra sludge volume was discharged manually from the bioreactor every day. The discharged volume was replaced with the synthetic wastewater. The initial part of the experiment due to a divergence in the behavior of microbial community from conventional activated sludge was conducted for 28 days.

Operational conditions for growth of AOB and NOB

The bioreactor was seeded with NAS. Initially, mixed liquor suspended solid was kept at 2000 mg/L. The pH of synthetic wastewater and the activated sludge was stabilized at 7–7.3 by supplying bicarbonate salt ($NaHCO_3$). The bioreactor was fed with synthetic wastewater including 40 mg/L $N–NH_4^+$ and sufficient alkalinity (5.95 g $NaHCO_3$) during 75 days. The synthetic wastewater contains $(NH_4)\cdot SO_4$, $NaH_2PO_4\cdot H_2O$, $NaHCO_3$, and 1 L of trace metal solution: EDTA (10 mL), $ZnSO_4\cdot 7H_2O$ (2.20 mL), $CoCl_2\cdot 6H_2O$ (3.20 mL), $MnCl_2\cdot 4H_2O$ (10.20 mL), $CuSO_4\cdot 5H_2O$ (0.22 mL) $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ (2.20 mL), $CaCl_2\cdot 2H_2O$ (1.10 mL), $FeSO_4\cdot 7H_2O$ (10.00 mL), H_3BO_3 (0.30 mL), $NiSO_4\cdot 6H_2O$ (1.00 mL). In order to enhance nitrification efficiency, C/N ratio in the feed was retained at zero. Variations of all nitrogen compounds, as well as microbial metabolites, were monitored daily. In order to monitor the growth and metabolite building up through AOB and NOB, a control experiment enriched with CAS was conducted along with NAS. Additional information regarding the control experiment is located in the related paper (Sepehri and Sarrafzadeh 2018).

Determination of growth parameters using CO_2 uptake and nitritation and nitratation rate: validation strategy for enrichment of AOB and NOB in attributed phases

In this study, the growth parameters including μ_{max} and Y_A were calculated based on the results of the titrimetric and offgas analyzer (TOGA) method proposed by Blackburne et al. (2007). At the end of each phase, the sludge was transferred to TOGA. Briefly, TOGA has a closed reactor filled with target biomass at the end of each phase of growth. Titrimetric pH meter controls acidity in the system by adjusting hydrochloric acid and sodium hydroxide dosing pump. The amount of acid/base usage in the reactor is recorded as the rate of proton accumulation in the processes occurring in bioreactor. The measure of gas concentration in feed gas along with offgas leads in gas components transfer rates in the gas phase mass balance. Oxygen transfer rate (OTR) can be used when the transfer of oxygen reaches equilibrium. The carbon dioxide uptake rate (r_{CO_2}) is found by measured carbon dioxide transfer rate (CTR_{meas}) and hydrogen

ion production rate (HPR_{meas}). When HPR_{meas} is positive, it shows production of hydrogen ion. The below-mentioned equations show the mathematical equations for HPR_{meas} and r_{CO_2}:

$$r_{CO_2} = \left(HPR_{meas} - rH + \frac{r_{NH_3}}{1 + 10^{(pH-pKn)}}\right)(1 + 10^{(pKa-pH)} + CTR_{meas} \quad (1)$$

Below we summarized each rates for nitrification steps:

Nitritation process:

$$r_{NH_3} = \left(\frac{1}{Y_{A_1}} + i_{XB}\right) r_{A_1} \quad (2)$$

$$r_{O_2} = \left(\frac{1.5}{Y_{A_1}} - 1\right) r_{A_1} \quad (3)$$

$$r_{CO_2} = r_{A_1} = r_X \quad (4)$$

$$r_{NO_2^-} = \frac{1}{Y_{A_1}} r_{A_1} = r_{H^+} \quad (5)$$

Nitratation process:

$$r_{NH_3} = i_{XB} r_{A_2} \quad (6)$$

$$r_{O_2} = \left(\frac{0.5}{Y_{A_2}} - 1\right) r_{A_2} \quad (7)$$

$$r_{CO_2} = r_{A_2} = r_X \quad (8)$$

$$r_{NO_2^-} = \frac{1}{Y_{A_2}} r_{A_2} \quad (9)$$

$$r_{NO_3^-} = \frac{1}{Y_{A_2}} r_{A_2} \quad (10)$$

Nitrification process (Nitritation + Nitratation)

$$r_{NH_3} = \left(\frac{1}{YA} + i_{XB}\right) r_A \quad (11)$$

$$r_{O_2} = \left(\frac{2}{Y_A} - 1\right) r_A \quad (12)$$

$$r_{CO_2} = r_A = r_X \quad (13)$$

$$r_{H^+} = \frac{1}{Y_{A1}} r_{A1} \quad (14)$$

$$r_{NO_3^-} = \frac{1}{Y_A} r_A \quad (15)$$

Measurement techniques for substrates and metabolites in biological reactors

Samples were taken from the bioreactor at the beginning and end of every day and were immediately centrifuged for the analysis of $N–NH_4^+$, $N–NO_2^-$, $N–NO_3^-$, EPSc, EPSp, SMPc, SMPp, UAP, and BAP. The concentrations of MLSS, $N–NH_4^+$, $N–NO_2^-$ and $N–NO_3^-$ were measured in accordance with the standard methods for examination of water and wastewater (Rice et al. 2012). pH was monitored using a pH probe (240, ISTEK, Korea), and dissolved oxygen (DO) was monitored by DO sensor (WTW340i, Germany). In order to determine the amount of $N–NH_4^+$, $N–NO_2^-$, and $N–NO_3^-$, Agilent 8453 UV–Vis spectrophotometer was utilized at 640 nm for NH_4^+, at 275 and 220 nm for NO_3^-, and at 543 nm for NO_2^-.

Protein parts (EPSp, SMPp) and carbohydrate parts (EPSc, SMPc) of SMP and EPS were measured based on the method proposed by Le-Clech et al. (2006). The SMP values in the supernatant were measured through consecutive centrifuging of microbial samples at 5000 g for 5 min and filtration process of the remaining supernatant. The mixing process was done after adding deionized water to the samples for 10 min. A series of heating and centrifuging processes were put and placed in order to remove impurities. The heating and centrifuging processes were carried out at 80 °C and 7000 g during a 10-min span. The EPS content in both forms was measured through supernatant filtration process.

The fresh activated sludge sample was washed and transferred to another bioreactor equipped with a heater in order to maintain temperature around 20 °C. BAP was measured without the addition of substrate, whereby BAP dominated microbial products as proposed by Jiang et al. (2008). Thirty milliliter of sludge sample was collected from the bioreactor and soluble COD and EPS measured daily. The BAP production rate was calculated from the variation in EPS concentration. In the direction of quantifying the UAP content values, the sludge was spiked with 2 L of ultra-filtered wastewater. The wastewater was filtered through a 0.45-µm membrane. Next, the wastewater underwent an ultrafiltration membrane (PLAC cellulosic disks, MWCO, 1000 Da, Millipore Inc., USA) in order to concentrate the soluble degradable substrate (Xie et al. 2016).

Results and discussion

Detecting the growth of AOB and NOB via a consecutive boom and bust cycle and nitrogen transformation

$N–NO_2^-$, intermediate compound in nitrification and a dependable parameter quantifying the intensity and degree of nitritation and nitratation, was detected as a response. Nitrite is recognized as an unstable nitrogen compound and can easily be converted to nitrate. Various studies have shown that the accumulation of nitrite in the environment is rarely observed because of two reasons: (1) a higher substrate utilization rate of nitrite oxidizers than ammonia oxidizers and (2) a low S_{min} (minimum required substrate concentration in order to support microbial community) (Kim et al. 2008). Nitrite accumulation was observed in some specific environmental conditions such as high temperature (Kim et al. 2008). Variation of $N–NH_4^+$, $N–NO_2^-$ and $N–NO_3^-$ is shown in Fig. 2. It must be taken into account that the initial concentration of $N–NH_4^+$ and $N–NO_3^-$ was 40 and 10 mg/L, respectively. The results in Fig. 2 yielded some valid findings, which consisted of two stages. The datasets for the first stage span the period from zero to the 5th day and for the second stage from 5th to 7th day. A cursory glance at Fig. 2 reveals that a particular pattern occurred in the bioreactor. A reasonable explanation for the periodical cycle could be based on the first step in nitrification. The overall downward trend in $N–NH_4^+$ species concentrations confirms the effective occurrence of nitritation process throughout the entire operation of the bioreactor. The maximum amounts of nitrite build-up are 30, 33, 34 and 35 mg/L which occurred in 5th day of each week. The trend of nitrite accumulation correlates favorably with Kim et al. (2008) and further supports the idea that nitrifiers were activated and dominated in NAS and making the results to be interpretable based on nitrite-dependent point of view. The observed increase in nitrite until the 5th day in each week could be attributed to the dominant activity of AOB (seen as the shoulder) since the nitritation process leads to nitrite accumulation. According to Fig. 2, there exists a negative correlation between ammonium and nitrite concentration in nitritation-dominant period. A reasonable explanation for such correlation might be the complete ammonium removal. AOB were able to convert all ammonium before the 5th day of the last months of enrichment. According to Fig. 2, in the 6th day, AOB did not have access to ammonium. Similarly, the observed decrease in nitrite from 5th to the 7th day could be interpreted as being a result of the dominant activity of NOB since the accumulation of both nitrite and nitrate confirms their activity. Final nitrite concentrations at the end of each batch are 3.76, 3.22, 2.64 and 1.79 mg/L. In the five first days of each week in view of the fact that there was not enough nitrite production, the NOB cannot acquire essential energy from the oxidation of little-produced nitrite. Due to nitrite production by AOB, the nutritional condition was proper for the activity of NOB. After the 5th day, NOB completely converted all of the nitrite to nitrate. It was depicted above that the amount of nitrite production was largely determined by the amount of ammonium concentration in the reactor. Dytczak et al. (2008) analyzed the influence of nitrite concentration on ammonium utilization rate in batch condition and demonstrated up to 35 mg $N–NO_2^-$ caused no inhibition for activity of AOB. According to Dytczak results, nitrifier can prolong nitritation and nitratation periodical cycles without suppression due to nutrient shocks. The data provide convincing evidence demonstrating that nitrite is a key component of a link between AOB and NOB. According to the nitrite results, reaching to maximum nitrite takes 5 days and

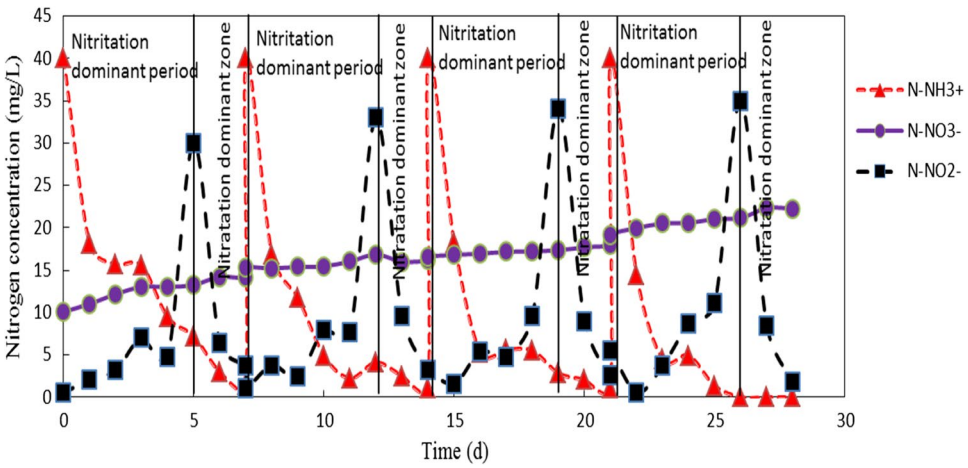

Fig. 2 Monitoring the symbiosis interaction of AOB and NOB through the non-molecular approach (consecutive boom and bust cycles): $N–NO_2^-$ along with $N–NH_4^+$ and $N–NO_3^-$ profiles can elucidate the enrichment of nitrifiers in a sludge sample

reaching to minimum nitrite takes 2 days in each week, which shows the longer nitritation than nitratation process. The longer nitritation process is consistent with the literature. Kim et al. (2008) found that in the temperature ranges 20–30 °C activation energy of nitritation is 87.1 kJ mol^{-1} and activation energy for nitratation is 38.6 kJ mol^{-1}. According to the Kim results, nitritation is the rate-limiting step that the nitrite trend in Fig. 2 validates their results. Kowalchuk and Stephen (2001) also studied the microbial behavior of AOB and mentioned chemolithoautotrophic AOB are responsible for the rate-liming step of nitrification in a variety of environments. Collectively, these findings implicate evidently the distinguishable phases for the dominant activity of AOB and NOB during the batch tests. These consecutive cycles provide an authentic methodology to quantify the growth of AOB and NOB distinctively. In addition, the observed production and consumption of nitrite along with parallel ammonium utilization and nitrate generation in oxic period can be assigned to the collaboration between AOB and NOB. The repeated cycle of nitrite occurred in NAS is called "Boom and Bust" or "feast and famine" in ecological science.

"Boom" is characterized by the rapid growth and activity of one species that is followed by "Bust" during which the activity of dominant species falls back to a minimal level. The noticeable depletion of ammonium concentration and production of nitrate through the occurrence of the boom and bust cycle promote the idea that AOB and NOB are the dominant active species in the community. Microbial community in NAS provided a comparable response during boom and bust cycle. Based on current data, for the first 5 days, aerobic nitrifiers consistent with *Nitrosomonas* are better competitor, while for two final days of each phase the alternating bacteria identified as *Nitrobacter* would proliferate.

Evaluation of dominant period in terms of alkalinity consumption

Nitrification is a well-known biological reaction in alkalinity consumption (Sepehri and Sarrafzadeh 2018). As a result, pH of activated sludge reduces. In order to validate the dominant period, alkalinity consumption was measured. From 0 day to 5th day, 5.2 g $NaHCO_3$ was used on average through 4 weeks. On contrary, from 5th day to 7th day, 1.3 g $NaHCO_3$ was used on average through 4 weeks to compensate alkalinity requirement for biological activity. Comparison between these values for alkalinity consumption substantiates the division between nitritation-dominant period and nitratation-dominant period. Theoretically, alkalinity consumption through nitritation is higher than nitratation since there is not hydrogen ion produced through nitratation (Peng and Zhu 2006).

Determining Y_A and μ_{Amax} from nitrogen oxidation rate

The main kinetic coefficients for microbial growth measurements are maximum growth rate (μ_{Amax}) and yield (Y_A). The presence of boom and bust cycles can distinguish the growth parameters related to both AOB and NOB independently. To study nitrifiers growth more precisely, values for μ_{Amax} and Y_A were calculated. Nitrifiers growth parameter was calculated according to the method proposed by Blackburne et al. (2007) through the rate of carbon dioxide uptake and rate of oxygen rate and NH_4^+ (or nitrite) utilization. Table 1 summarizes the obtained values in the current study and values quoted in the literature. Independent phases represent different weeks in which growth of nitrifiers occurred. μ_{Amax} and Y_A values demonstrated in Table 1 concur well with Melcer (2004) findings regarding the temperature-adjusted values to 30 °C applying the coefficient $1.076^{(T-20)}$. The consistency of growth parameters values with the previous

Table 1 Growth parameters of AOB and NOB after nitrifiers enrichment during 75 days. Values were determined at temperature of 30 °C

Experiment number	μ_{Amax} ($days^{-1}$) (AOB)	Y_A (gVSS gN^{-1}) (AOB)	μ_{Amax} ($days^{-1}$) (NOB)	Y_A (gVSS gN^{-1}) (NOB)	T_d (day)
Phase 1: (0–7th day)	0.510	0.16	0.683	0.072	1.66
Phase 2: (7th–14th day)	0.523	0.17	0.632	0.062	1.62
Phase 3: (14th–21th day)	0.440	0.18	0.630	0.063	1.58
Phase 4: (21th–28th day)	0.670	0.17	0.710	0.072	1.50
Vadivelu et al. (2006a, b) (30 °C)	1.000	0.12	0.480	0.071	n.a.
Keen and Prosser (1987) (30 °C)	0.936	n.a.	1.032	n.a.	n.a.
Blackburne et al. (2007)	0.48–0.65	0.11–0.18	0.66–0.68	0.062–0.081	n.a.

The values for phase 1 to phase 4 correspond to the mean value adjusted to 30 °C applying the relation $1.079^{(T-20)}$

n.a. Not available in the literature

study in the literature suggests feasibility and efficacy of the defined method with full-scale activated sludge. The presence of a subtle difference in the obtained values and literature values can be due to operational parameters. A significant difference was associated with the contrast in two important parameters including temperature and pH. These parameters profoundly influenced the growth rate of AOB and NOB. Vadivelu et al. (2006a b) determined the value of $Y_A = 0.071$ gVSS gN^{-1} and $\mu_{Amax} = 0.48$ $days^{-1}$ for NOB enriched community at temperature 22 °C, pH 7.3. According to the Water Research Commission (Ekama et al. 1984), the correction factor for pH-adjusted values is $2.35^{(pH-7.2)}$. They designed activated sludge processes for nutrient removal. In this particular study due to the growth of nitrifiers bacteria, the factor for pH correction was $2.9^{(pH-7.1)}$. Another explanation for the difference in growth parameter can also be attributed to either the production of microbial metabolites such as SMP and EPS or the energy requirement of cell maintenance (Fang et al. 2009). An additional source of error might be accumulation of carbon dioxide/bicarbonate in nitrifiers community in order to achieve the bicarbonate equilibrium which results in generation of protons into the activated sludge (Blackburne et al. 2007).

Microbial metabolites generated by the activity of AOB and NOB in the consecutive boom and bust cycles

SMP and EPS production by AOB and NOB

The minimum C/N ratio and thereafter AOB and NOB abundance in bioreactor displayed significant influence onto the sludge EPS, and bulk supernatant SMP. In order to investigate the process in terms of metabolite production, the profiles of EPS and SMP are illustrated in Figs. 3 and 4. Extracellular protein concentration (Fig. 3a) increased in either nitritation or nitratation processes during 28 days of growth. AOB under $N–NH_4^+$ enriched conditions yielded 0.95 mg/L EPSp after 28 days of growth compared to 0.4 mg/L for

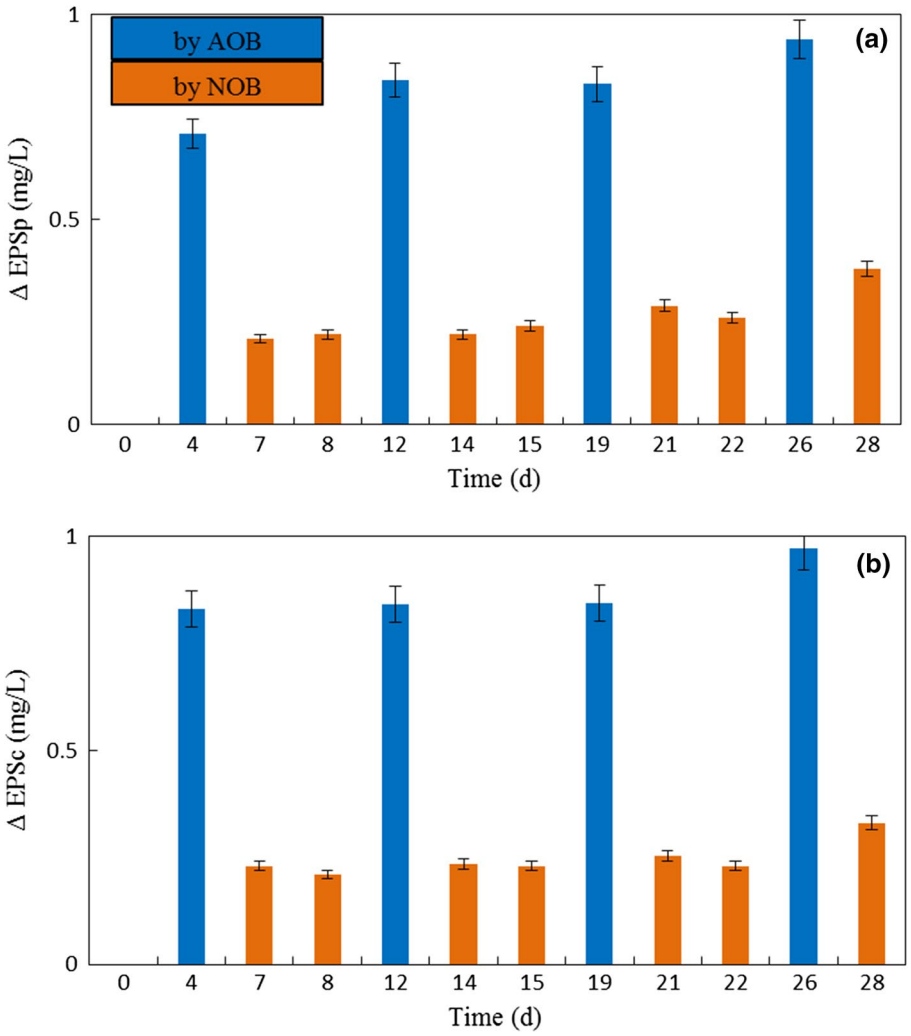

Fig. 3 Quantitative analysis of microbial metabolite synthesis of AOB and NOB under boom and bust cycle after enrichment: **a** variation in ΔEPSp and total EPSp generation, **b** variation in ΔEPSp and total EPSp generation

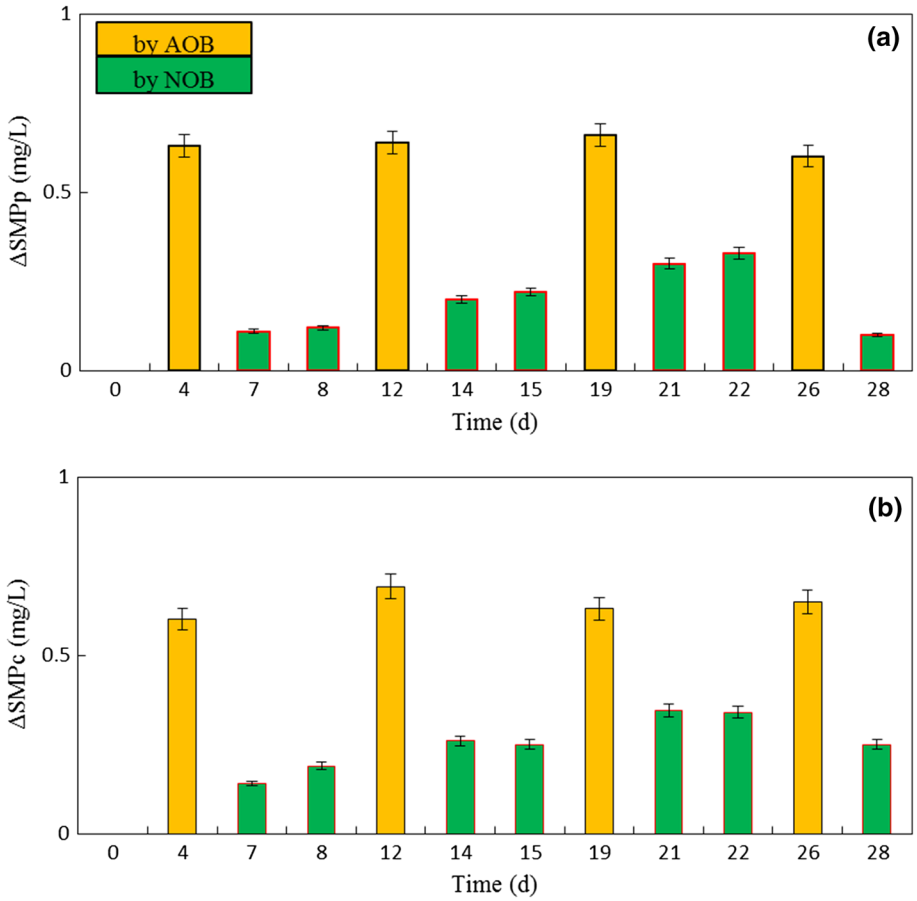

Fig. 4 Bulk supernatant soluble microbial products and their fraction in batch test after AOB and NOB enrichment through non-molecular approach: **a** variation in ΔSMPp and total SMPp generation. **b** Variation in ΔSMPc and total SMPc generation

N-NO_2^- enriched conditions. The specific production of EPS, including protein and carbohydrates, was more than that of SMP. When AOB abundance increased continuously, the ΔEPSp content increased from 0.7 to 0.92 mg/L. The ΔEPS and ΔSMP and their component fractionalized as carbohydrates and protein-like substances in the bioreactor were relatively stable during the whole experiment time. Compared with the relatively stable ΔEPS content by AOB and NOB, a continuous elevation of ΔSMPc as well as ΔSMPp content with an increase of AOB and NOB community occurred in the bioreactor (Fig. 4a, b). However, the elevation of ΔSMPc as well as ΔSMPp content for AOB was substantially higher than NOB in nitrifiers-enriched activated sludge. The probable reason for higher metabolite production by AOB might be related to the kinetic of nitritation and nitratation processes. Soluble microbial products (SMP) and extracellular polymeric substances (EPS) generated by AOB were 1.2 and 1.8 mg/L, respectively, while NOB produced 0.6 mg/L of SMP and 1 mg/L of EPS.

The kinetics of the second step of nitrification could be controlled by different parameters such as availability of substrates, oxygen concentration (shear rate), and SRT (Mertoglu et al. 2006). However, the very different amounts of nitrite also affect the nitrifying community. In the bioreactor with nitrite accumulation shows that oxidation rate by AOB was faster than NOB. Therefore, there is the least time for NOB to exert EPS and SMP. Jia et al. (1996) investigated the influence of cultivation time on EPS generation in activated sludge and showed that EPS level could be closely related to the microbial growth phase. Here, the cultivation time, according to the all nitrogen variation compounds can confirm higher cultivation time for AOB compared to NOB. Another probable reason for a higher metabolite of AOB might be due to the shear rate of the bioreactor. The shear rate or aeration intensity could have more influence on the EPS content in sludge (Adav et al. 2008). Oxygen concentration influences the distribution of AOB and NOB in environmental systems. Schramm et al. findings proved oxygen concentration can be a limiting factor manipulating activity and abundance of nitrifiers. Schramm et al. (1996) demonstrated dependability of *Nitrosomonas* and *Nitrobacter* species on oxygen through K_m (substrate affinities by Michaelis–Menten kinetics) values for oxygen. In addition, nitrifiers establish rather compact and dense colonial structure. Schramm et al. concluded that under low oxygen tension, NOB was out-competed by AOB. In another study, Okabe et al. (1999) observed that AOB strains can tolerate the low oxygen concentration. According to the

above-mentioned researches, the availability of oxygen for NOB was lower than AOB and resulted in an environmental stress. Therefore, AOB metabolism that was higher than NOB led to a larger portion of organic metabolite synthesis. Another reason for higher metabolite generation by AOB may be due to the limited SRT. Xie et al. (2013) showed the positive correlation between organic released metabolites values and SRT.

Quantification of UAP and BAP by AOB and NOB

Variations of UAP and BAP were monitored in order to have a clearer view of AOB and NOB microbial behavior. Significant progress has been recently made to differentiate quantitatively and accurately UAP and BAP. Characterization of UAP and BAP was conducted according to the unified theory. According to this theory, UAP are built up during assimilation, and BAP are built up through hydrolysis of biomass content (Ni et al. 2011). Formation of BAP is achieved at a rate proportional to the EPS concentration. Total EPS hydrolysis coefficient determines the amount of BAP produced by AOB and NOB. The UAP and BAP content values of the AOB and NOB reported, compared well with values in the literature (Xie et al. 2012). The BAP content of sludge samples was observed to be higher than that of UAP. In order to validate UAP and BAP values, control experiments with conventional activated sludge process were done in the previous study. The results of EPSt and SMPt generation via conventional activated sludge enriched with heterotrophs (Fig. 5) demonstrated the substantially higher amount of metabolites than generated by nitrifiers (Sepehri and Sarrafzadeh 2018). As can be seen in Fig. 6, BAP concentration increased steadily and linearly from 1.3 to 2.6 mg/L and formed the main component of SMP mainly after the first week. BAP accounts for more than 92% of SMP on average. The values correlate satisfactorily with Xie et al. (2012) and further support the idea that metabolites of AOB and NOB are much lower than heterotrophic bacteria. Figure 6 demonstrates that there exists four consecutive nitritation and nitratation process during the whole experiment. The produced amount of UAP and BAP by NOB initially was similar to AOB. After 2 weeks, these values for AOB exceeded from NOB. While NOB were low in utilization-associated products (UAP) (0.07 mg/L) and biomass-associated products (BAP) (0.12 mg/L), AOB were higher in UAP (0.15 mg/L) and BAP (0.3 mg/L). One of the significant differences in the values of UAP and BAP could be due to the initial source of their generation. Since the source of UAP is carbonaceous compounds from the substrate and the source of BAP cellular macromolecules containing organic carbon and nitrogen, generation of BAP is expected to be higher (Jarusutthirak and Amy 2006; Merkey et al. 2009). In addition, the colonies of NOB are mainly less dense than AOB cluster and in general NOB colonies and cells are more uniformly distributed compared to AOB colonies. These values offer overwhelming evidence for higher metabolism and metabolite production by AOB. The results suggest that moving the microbial community toward NOB enrichment and confining the growth of AOB can lead to lower organic metabolite in the effluent.

Barker and Stuckey (1999) demonstrated that through closing a mass balance on SMP using radio-active ^{14}C tracer, BAP generation is related to the biomass decay stoichiometrically. Nevertheless, their assessment failed to take into account the EPS component of biomass. The results demonstrate the importance of reduction of metabolites and addressing the parameters influencing the production of the metabolites. One of the governing

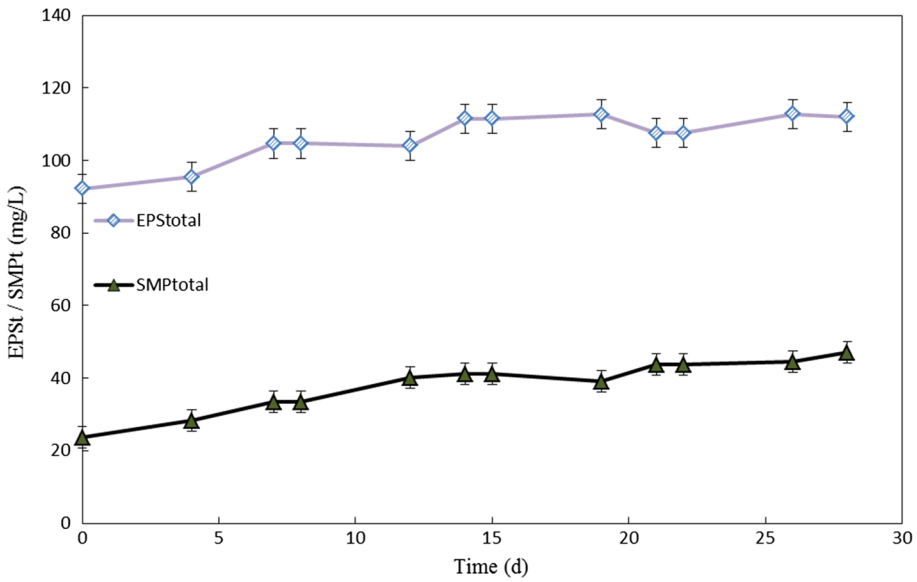

Fig. 5 Generation of SMPt and EPSt in the conventional activated sludge enriched with heterotrophs

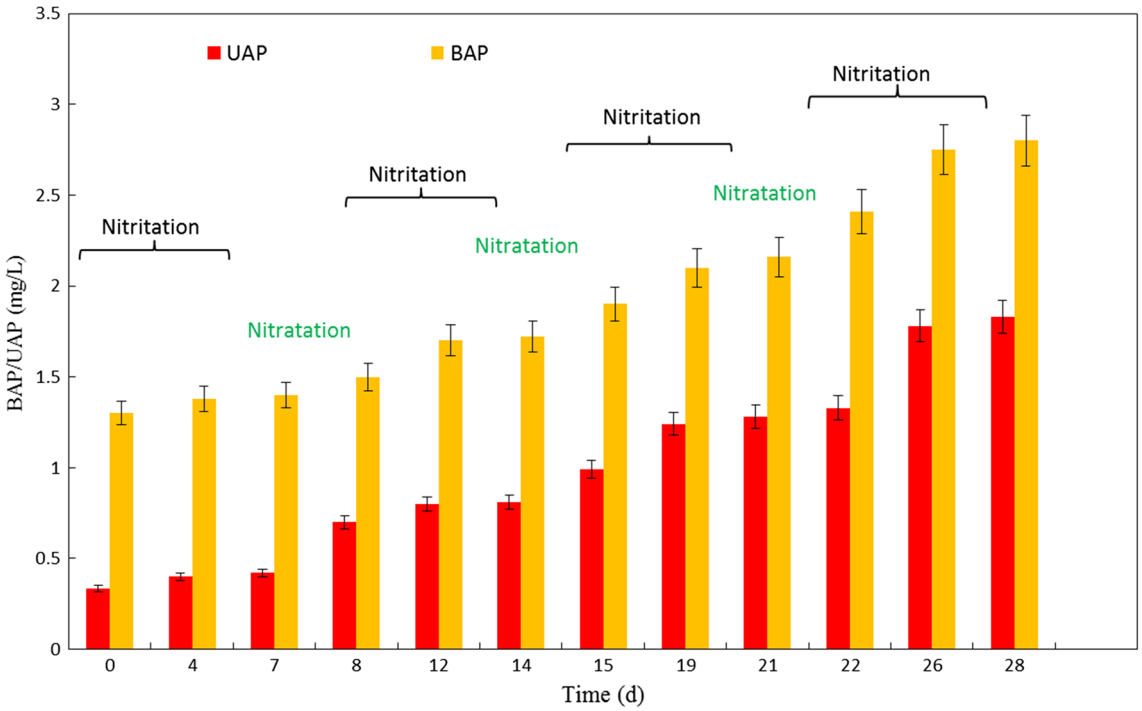

Fig. 6 Responses of BAP, UAP, total SMP, and total EPS through nitritation-dominant period and nitratation-dominant period. The presence of positive correlation between BAP and nitrification and a negative correlation between UAP and nitrification process elucidate the symbiosis interaction of AOB and NOB in batch mode

parameters influencing on UAP and BAP production could be SRT. A longer SRT might promote the generation of UAP and BAP since SMP and EPS production could have positive correlation with SRT. Dependability of UAP formation on SRT values could be lower than BAP formation in autotrophic conditions (Xie et al. 2016). Okabe et al. (1999) spatial distribution results revealed that AOB were detected throughout the biofilm, while the NOB mainly were detected in the inner parts of both conventional activated sludge and nitrifying enriched activated sludge. Due to the distinct spatial distribution of AOB and NOB in microbial biofilm, there exists a NO_2^- peak concentration in the surface of the biofilm which is in positive correlation with NO_2^- concentration in bulk. Their results also are in agreement with the nitrite profile that was discussed in the previous section. As a result, one the main reason for the higher production of associated products by AOB than NOB could attribute to different environmental stresses inside the nitrifying biofilm.

The emerging application of NOB intensification due to lower metabolite generation in comparison with AOB can be used in the advance wastewater treatment processes such as MBR that suffers from biofouling. The NOB-developed approach not only can mitigate biofouling dramatically but also can reduce the amount of by-products since these metabolites are precursors for chlorinated organic compound such as trihalomethanes in tertiary treatment.

Conclusion

In this study, monitoring the growth of AOB and NOB was performed through the development of a viable non-molecular approach. To control the microbial metabolites, a method was recommended which is strengthened by nitratation intensification process. Nitrite as an intermediate component, which links AOB and NOB, was detected during 28 days after enrichment. The presence of boom and bust cycle (feast-famine cycle) in nitrogen transformation through cultivation-dependent strategy demonstrates the enrichment as well as the proportion of AOB and NOB in nitrifiers community. The developed strategy can quantify the exerted organic metabolites such as EPS and SMP in microbial-involved wastewater treatment processes. BAP generations were remarkably higher than UAP in either nitritation or nitratation. The portions of AOB in microbial products were substantially higher than NOB, which might prove the far higher duty of cooperation to supply heterotrophic feed in activated sludge-involved process. The perfect positive correlation between AOB and microbial metabolites introduced an approach, and

control of C/N ratio in boom and bust cycle can prolong the nitratation process.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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