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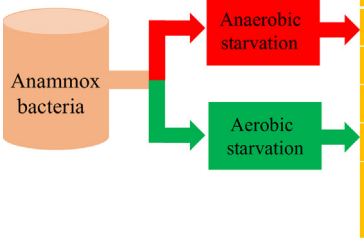
HIGHLIGHTS

- Death and activity decay of Anammox bacteria were distinguished.
- Decay, death and activity decay rates of Anammox bacteria were determined.
- Death was mainly responsible for the decreased Anammox activity during starvation.
- Activity decay played a minor role in the decreased Anammox activity.

GRAPHICAL ABSTRACT



Parameter	Anaerobic starvation	Aerobic starvation
Decay rate (d^{-1})	0.015±0.001	0.028±0.001
Death rate (d^{-1})	0.011±0.001	0.025±0.001
Activity decay rate (d^{-1})	0.004±0.001	0.003±0.001
Contribution of death (%)	73±4	89±4
Contribution of activity decay (%)	27±5	11±5

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ABSTRACT

The decreased activity (i.e. decay) of anaerobic ammonium oxidation (Anammox) bacteria during starvation can be attributed to death (i.e. decrease in the amount of viable bacteria) and activity decay (i.e. decrease in the specific activity of viable bacteria). Although they are crucial for the operation of the Anammox process, they have never been comprehensively investigated. This study for the first time experimentally assessed death and activity decay of the Anammox bacteria during 84 days' starvation stress based on ammonium removal rate, Live/Dead staining and fluorescence in-situ hybridization. The anaerobic and aerobic decay rates of Anammox bacteria were determined as 0.015 ± 0.001 d^{-1} and 0.028 ± 0.001 d^{-1}, respectively, indicating Anammox bacteria would lose their activity more quickly in the aerobic starvation than in the anaerobic starvation. The anaerobic and aerobic death rates of Anammox bacteria were measured at 0.011 ± 0.001 d^{-1} and 0.025 ± 0.001 d^{-1}, respectively, while their anaerobic and aerobic activity decay rates were determined at 0.004 ± 0.001 d^{-1} and 0.003 ± 0.001 d^{-1}, respectively. Further analysis revealed that death accounted for $73 \pm 4\%$ and $89 \pm 5\%$ of the decreased activity of Anammox bacteria during anaerobic and aerobic starvations, and activity decay was only responsible for $27 \pm 4\%$ and $11 \pm 5\%$ of the decreased Anammox activity, respectively, over the same

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starvation periods. These deeply shed light on the response of Anammox bacteria to the starvation stress, which would facilitate operation and optimization of the Anammox process.

1. Introduction

In the wastewater treatment plants (WWTPs), bacteria are frequently exposed to the starvation conditions because of the large fluctuations in the wastewater flow and composition. Under such starvation conditions, bacteria could switch on programmed cell death (Lewis, 2000; Yarmolinsky, 1995), which is a genetically determined cell self-destruction process, to maintain partial bacterial activity. This results in bacteria death (i.e. decrease in the amount of viable bacteria). In addition, bacteria could also adjust their metabolic processes and decrease their maintenance energy requirement under starvation conditions (Boutte and Crosson, 2013; Durfee et al., 2008). In that case, bacteria would be self-controlled via enzymatic regulation instead of being dead, which leads to activity decay (i.e. decrease in the specific activity of viable bacteria). Bacteria death and activity decay collectively contribute to the decreased bacterial activity (i.e. bacteria decay) in the starvation condition (Hao et al., 2009, 2012; van Loosdrecht and Henze, 1999).

Bacteria decay will significantly affect the robustness and performance of WWTPs. Therefore, plenty of researches have been conducted to measure the decay rates of key bacteria/archaea involved in wastewater treatment, such as ammonium-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB), heterotrophic bacteria, polyphosphate-accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs) and methanogens (Carvalheria et al., 2014; Hao et al., 2009, 2012; Salem et al., 2006; Vargas et al., 2013). For instance, the decay rates of AOB and NOB were determined as 0.02 d^{-1} and 0.08 d^{-1}, respectively, by Salem et al. (2006). In addition, Hao et al. (2009, 2010a, 2012) also further experimentally evaluated the death and activity decay of AOB, NOB, PAOs, GAOs, heterotrophic bacteria and methanogens.

In addition to the above bacteria/archaea, anaerobic ammonium oxidation (Anammox) bacteria are also of great importance to wastewater treatment (Kartal et al., 2010; Xu et al., 2015). The partial nitritation and Anammox process has revolutionized the way of nitrogen removal (Kartal et al., 2010; Lotti et al., 2015; Xu et al., 2015). In this process, AOB first oxidize about half of the ammonium to nitrite, while the Anammox bacteria subsequently convert nitrite and the remaining ammonium to N_2. Compared to traditional nitrogen removal via nitrification and denitrification, the partial nitritation and Anammox process has lower energy consumption, negligible organic carbon requirement and much less sludge production (Kartal et al., 2010; Lotti et al., 2015; Xu et al., 2015). More than one hundred full-scale Anammox plants have already been operated for the treatment of municipal and industrial wastewaters until now (Lackner et al., 2014). Unfortunately, the aerobic decay rate of Anammox bacteria has never been measured so far although the anaerobic decay rate of Anammox bacteria has been measured by many researchers (Ma et al., 2016; Scaglione et al., 2009; Xing et al., 2016a; Zhang et al., 2015). Determining aerobic decay rate of Anammox bacteria is crucial because the Anammox bacteria could also be exposed to the aerobic starvation condition at a dissolved oxygen (DO) concentration of up to 1.5 mg/L (Lackner et al., 2014). In addition, due to the lack of a suitable approach, previous studies did not differentiate death from activity decay (Scaglione et al., 2009; Xing et al., 2016a; Zhang et al., 2015). Therefore, several questions have been raised. Do the death and activity decay of the Anammox bacteria happen simultaneously or sequentially under starvation conditions? What is the contribution of death and activity decay to the decreased activity of Anammox bacteria? These questions have not been answered so far.

By addressing the above questions, this study aims to experimentally assess death and activity decay of the Anammox bacteria under both anaerobic and aerobic starvation conditions. The enriched Anammox culture was exposed to the anaerobic and aerobic starvation for 84 days, during which Live/Dead staining and fluorescence in-situ hybridization (FISH) were conducted periodically to determine the ratios of viable bacteria and viable Anammox bacteria, respectively. The activity of Anammox bacteria was also monitored over the same period via a series of batch tests. The anaerobic and aerobic decay rates, death rates and activity decay rates of the Anammox bacteria were then determined and assessed by the detailed analysis of the comprehensive experimental data. The contributions of death and activity decay to the decreased Anammox activities were also calculated.

2. Materials and methods

2.1. Cultivation of Anammox bacteria

One pilot-scale up-flow bioreactor with a working volume of 50 L was set up to cultivate Anammox bacteria. The bioreactor was operated at 33 ± 2 °C with a pH of 7.5 ± 0.3 and a DO of approximately 0 mg/L. The bioreactor received synthetic wastewater (sparging N_2 regularly) continuously, which contained 1180 mg $(NH_4)_2SO_4$/L (i.e. 250 mg N/L), 1230 mg $NaNO_2$/L (i.e. 250 mg N/L), 540 mg $NaHCO_3$/L, 32 mg KH_2PO_4/L, 216 mg $CaCl_2 \cdot 2H_2O$/L, 360 mg $MgSO_4 \cdot 7H_2O$/L, 0.018 mg H_3BO_3/L, 0.275 mg $NaMoO_4 \cdot 2H_2O$/L, 0.3 mg $CoCl_2 \cdot 6H_2O$/L, 3.75 mg EDTA/L, 0.24 mg $NiCl_2 \cdot 6H_2O$/L, 0.25 mg $MnCl_2 \cdot 4H_2O$/L, 0.54 mg $ZnSO_4 \cdot 7H_2O$/L and 0.31 mg $CuSO_4 \cdot 5H_2O$/L. The bioreactor was operated for 280 days (hydraulic retention time was 0.5 d) with a total nitrogen removal rate of approximately 630 mg N/L/d and an Anammox bacteria population of around 83% while reaching steady state. AOB were undetectable.

2.2. Starvation tests

3.0 L of Anammox sludge was taken out from the pilot-scale up-flow bioreactor when it reached steady state. Afterwards, the Anammox sludge was washed using the ammonium and nitrite free synthetic wastewater (see Section 2.1) for 5 times until no ammonium or nitrite were detectable. Then, the Anammox sludge was equally transferred into two starvation reactors (i.e. Erlenmeyer flask, 1.5 L each) to initiate the batch-mode starvation tests. One starvation reactor was operated under the anaerobic condition. The other starvation reactor was operated under the aerobic condition with a DO concentration of ~1.5 mg/L, which was the highest DO concentration reported in literature for the Anammox reactor (Lackner et al., 2014). pH in both starvation reactors was at approximately 7.5 ± 0.3 and the temperature was 33 ± 2 °C. The starvation tests lasted for 84 days, during which Live/Dead staining and FISH were conducted regularly. The mixed liquor volatile suspended solids (MLVSS) concentration was also monitored

periodically.

2.3. Batch tests to determine the activity of Anammox bacteria

During the starvation period, a series of batch tests were conducted regularly to determine the activity of the Anammox bacteria. Briefly, 100 ml of Anammox sludge was withdrawn from each starvation reactor on 0, 3, 18, 38, 58 and 84 d, and then washed using the ammonium and nitrite free synthetic wastewater (see Section 2.1). Afterwards, the Anammox sludge was added into two batch reactors (100 ml each). Following that, the sodium nitrite and ammonium sulphate stock solutions (both at 4.0 g N/L) were added into the batch reactors to achieve initial nitrite and ammonium concentrations of 50 mg NO_2^--N/L and 50 mg NH_4^+-N/L, respectively. During the activity measurement, pH, DO and temperature were at 7.5 ± 0.3, ~0 mg/L and 33 ± 2 °C, respectively. The mixed liquor samples were taken every 60 min for the analysis of ammonium. The activity tests lasted for 3 h. At the end of each activity test, the MLVSS concentration in each batch reactor was measured. The Live/Dead staining and FISH were also performed.

2.4. Determining decay, death and activity decay rates of Anammox bacteria

The decay rate of the Anammox bacteria was determined by fitting the measured volumetric Anammox activity to the natural logarithmic function (Eq. (1)) (Scaglione et al., 2009; Zhang et al., 2015). The volumetric Anammox activity was expressed as the volumetric ammonium removal rate, which was calculated through linear regression of the ammonium profile measured during the batch tests.

$$b = \ln(R(0)/R(t))/t \quad (1)$$

where b is the decay rate of Anammox bacteria (d^{-1}); R(0) is volumetric Anammox activity on Day 0 (mg N/L/h); R(t) is volumetric Anammox activity at time t (mg N/L/h); t is starvation time (day).

The death rate of the Anammox bacteria was calculated by fitting the concentration of viable Anammox bacteria to the natural logarithmic function (Eq. (2)).

$$Death = \ln(X_{viable}(0)/X_{viable}(t))/t \quad (2)$$

where Death is the death rate of Anammox bacteria (d^{-1}); $X_{viable}(0)$ is the concentration of viable Anammox bacteria on Day 0 (g/L); $X_{viable}(t)$ is the concentration of viable Anammox bacteria at time t (g/L).

The concentration of viable Anammox bacteria was calculated as follows (Eq. (3)) (Vadivelu et al., 2006; Hao et al., 2009, 2010a, 2012)

$$X_{viable} = MLVSS \times Live \times FISH \quad (3)$$

where MLVSS is the measured MLVSS concentration (g/L); Live is the ratio of viable bacteria to the total bacteria (i.e. viable + dead bacteria) (%) (i.e. Live/Dead staining results); FISH is the ratio of viable Anammox bacteria to the total viable bacteria (%) (i.e. FISH results) (e.g. Hao et al., 2009; Manser et al., 2006);

The activity decay rate of the Anammox bacteria was determined by fitting the specific activity of viable Anammox bacteria to the natural logarithmic function (Eq. (4)).

$$D_{activity} = \ln(SA_{viable}(0)/SA_{viable}(t))/t \quad (4)$$

where $D_{activity}$ is the activity decay rate of Anammox bacteria (d^{-1}); $SA_{viable}(0)$ is the specific activity of viable Anammox bacteria on Day 0 (mg N/g viable Anammox/h); $SA_{viable}(t)$ is the specific activity of viable Anammox bacteria at time t (mg N/g viable Anammox/h).

The specific activity of viable Anammox bacteria was calculated by dividing the volumetric ammonium removal rate by the viable Anammox bacteria concentration.

The contributions of death and activity decay to the decreased Anammox activity were calculated based on Eqs. (5) and (6).

$$C_{death} = Death/b \quad (5)$$

$$C_{activity} = D_{activity}/b \quad (6)$$

where C_{death} is the contribution of death to the decreased Anammox activity; $C_{activity}$ is the contribution of activity decay to the decreased Anammox activity.

2.5. Analytical methods

The analysis of MLVSS was conducted according to the standard methods (APHA, 2005). For the analyses of ammonium, nitrite and nitrate concentrations, the samples were filtered via disposable Millipore filter units (pore size: 0.22 μm) after sampling. Afterwards, their concentrations were measured by a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Wisconsin).

Live/Dead staining was performed to determine the ratio of viable bacteria to the total bacteria (i.e. viable + dead bacteria) using the Live/Dead® BacLight™ bacterial viability kits (Molecular Probes, L-7012), as detailed in Hao et al. (2009). Briefly, 1 ml of sludge sample was transferred into a 5 ml plastic tube together with 1.5 μl of SYTO® 9 (targeting both viable and dead bacteria) and 1.5 μl of PI (targeting dead bacteria), and then incubated in a dark place for 15 min at the room temperature. Afterwards, the viable bacteria will be stained with green fluorescence, whereas the dead bacteria will be stained with red fluorescence. For quantitative analysis, 20 random microscopic fields were taken for each sample using a confocal laser scanning microscope (Zeiss LSM 510 Meta, Germany). The ratio (biovolume basis) of viable bacteria was then determined via image analysis using DAIME version 1.3.

FISH was carried out to determine the ratio of viable Anammox bacteria to the total viable bacteria, as described in Daims et al. (2001). The following oligonucleotide probes were used (Schmid et al., 2003): FITC labelled EUB-mix (comprising EUB338, EUB338-II and EUB338-III) for most bacteria, and CY5 labelled Amx368 for Anammox bacteria. For quantitative FISH analysis, 20 random microscopic fields were taken for each sample using a confocal laser scanning microscope (Zeiss LSM 510 Meta, Germany). The ratio (biovolume basis) of viable Anammox bacteria was then determined via image analysis using DAIME version 1.3.

The significance of the results was assessed by an analysis of variance. $p > 0.05$ was regarded statistically insignificant, whereas $p < 0.05$ was regarded statistically significant.

3. Results and discussion

3.1. Decay rate of Anammox bacteria

The volumetric Anammox activities during the 84 days' anaerobic and aerobic starvation periods are shown in Fig. 1. They decreased ($p < 0.05$) gradually after starting the anaerobic and aerobic starvation. After fitting the volumetric Anammox activities to the natural logarithmic function (Fig. 1), the decay rates of Anammox bacteria over the anaerobic and aerobic starvation periods were determined as 0.015 ± 0.001 d^{-1} and 0.028 ± 0.001 d^{-1},

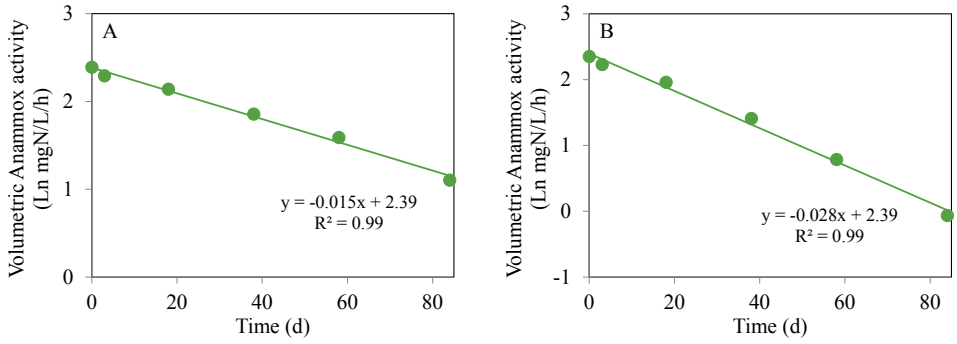

Fig. 1. Volumetric Anammox activities during the anaerobic (A) and aerobic (DO = 1.5 mg/L) (B) starvation periods. Solid lines represent the fit of the volumetric Anammox activities to the natural logarithmic function. Error bars indicate standard errors.

respectively (Table 1). These correspond to half-life times (t_H) of 46 d and 25 d (i.e. ln2 divided by decay rates) (Zhang et al., 2015). This is the first time that the aerobic decay rate of Anammox bacteria is determined, which would provide better insight into the performance of the Anammox system operated aerobically (Lackner et al., 2014). The decay rate in the aerobic starvation period is higher than that in the anaerobic starvation period, indicating that Anammox bacteria would lose their activity faster when exposed to the aerobic starvation condition. This is similar to AOB, NOB, PAOs, GAOs and heterotrophic bacteria, the aerobic decay rates of which are also higher than their anaerobic decay rates (Henze et al., 2000).

The determined anaerobic decay rate (0.015 ± 0.001 d^{-1}) in this study is in agreement with that (0.017 d^{-1}) reported in Xing et al. (2016b). But it is higher than those determined by Ni et al. (2009) (0.004 d^{-1}), Hao et al. (2002) (0.002 d^{-1}) and Xing et al. (2016a) (0.009 d^{-1}). The decay rates in Ni et al. (2009) and Hao et al. (2002) were estimated based on modelling instead of experimental measurement, which might result in the different values between our study and their studies. In the case of Xing et al. (2016a), the starvation occurred at 20 °C, which is lower than that (i.e. 33 °C) in our study. Xing et al. (2016b) have demonstrated that higher temperature would lead to a higher decay rate. Also, the decay rate in Xing et al. (2016a) was determined based on the decreased biomass (i.e. MLVSS) concentration, which did not consider the decay caused by the decreased specific activity (i.e. activity decay) of the Anammox bacteria. These collectively might lead to the lower decay rate in Xing et al. (2016a) in comparison with this study. Nevertheless, the decay rate in this study is much lower compared with that (0.128 d^{-1}) in Ma et al. (2016), in which the decay rate was determined through the decrease in the biomass-specific activity. As the biomass not only contained Anammox bacteria but also included heterotrophic bacteria growing on the lysate of bacteria, the determined decay rate might not accurately represent the decay rate of Anammox bacteria. This might contribute to the different decay rates.

Table 1
Anaerobic and aerobic decay parameters of Anammox bacteria (with standard errors).

Parameter	Anaerobic starvation	Aerobic starvation
Decay rate (d^{-1})	0.015 ± 0.001	0.028 ± 0.001
Death rate (d^{-1})	0.011 ± 0.001	0.025 ± 0.001
Activity decay rate (d^{-1})	0.004 ± 0.001	0.003 ± 0.001
Contribution of death (%)	73 ± 4	89 ± 4
Contribution of activity decay (%)	27 ± 5	11 ± 5

3.2. Death rate of Anammox bacteria

Fig. 2 shows the fractions of viable bacteria and viable Anammox bacteria in the anaerobic and aerobic starvation stages. The fractions of viable bacteria (relative to viable + dead bacteria) decreased ($p < 0.05$) from ~85% to ~50% and ~20%, respectively, over the anaerobic and aerobic starvation (Fig. 2A), revealing that aerobic starvation caused a higher bacteria death compared with anaerobic starvation. Microbial community analysis based on FISH demonstrated that the fraction of viable Anammox bacteria (relative to viable bacteria) did not change significantly ($p > 0.05$) and maintained at ~83% during the starvation period.

Based on the above results and MLVSS concentration, the viable Anammox bacteria concentration over the anaerobic and aerobic starvation periods were calculated and shown in Fig. 3. After initiating the starvation, the viable Anammox bacteria concentration decreased ($p < 0.05$) gradually. The experimentally determined viable Anammox bacteria concentrations were fit to a natural logarithmic function (solid lines in Fig. 3). Fig. 3 demonstrates that the natural logarithmic function well captured the measured data of viable Anammox bacteria concentration. The anaerobic and aerobic death rates of Anammox bacteria were then calculated as 0.011 ± 0.001 d^{-1} and 0.025 ± 0.001 d^{-1} (Table 1), respectively, which were smaller ($p < 0.05$) in comparison with the anaerobic and aerobic decay rates (0.015 ± 0.001 d^{-1} and 0.028 ± 0.001 d^{-1}) of Anammox bacteria. This confirms that the death of Anammox bacteria was only partially responsible for the decreased activity of Anammox bacteria over the starvation conditions.

This study for the first time determined the death rates of Anammox bacteria. Although Ma et al. (2016) also measured the anaerobic death rate of Anammox bacteria, they determined the death rate only based on the Live/Dead staining results and ignored the biomass decrease (i.e. MLVSS decrease) and the microbial composition (i.e. ignoring existence of heterotrophic bacteria). Therefore, the death rate in their study might not be able to reflect the true death rate of the Anammox bacteria. In addition, the aerobic death rate of Anammox bacteria is higher than their anaerobic death rate, which coincides with the trend of the decay rates of the Anammox bacteria.

3.3. Activity decay rate of Anammox bacteria

Fig. 4 shows the specific activity of Anammox bacteria over the anaerobic and aerobic starvation periods. Based on Fig. 4, the activity decay rates of Anammox bacteria were determined as 0.004 ± 0.001 d^{-1} and 0.003 ± 0.001 d^{-1}, respectively, in the anaerobic and aerobic starvation conditions (Table 1). This revealed that the anaerobic and aerobic activity decay rates of Anammox

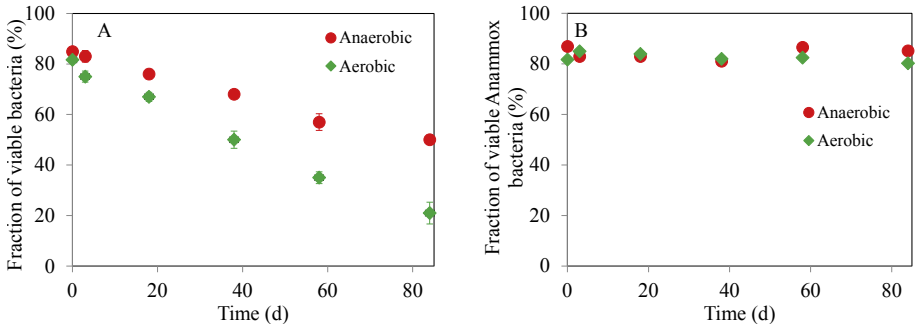

Fig. 2. Fractions of viable bacteria (A; relative to viable + dead bacteria) and viable Anammox bacteria (B; relative to viable bacteria) during the anaerobic and aerobic (DO = 1.5 mg/L) starvation periods based on Live/Dead staining and FISH quantification. Error bars indicate standard errors.

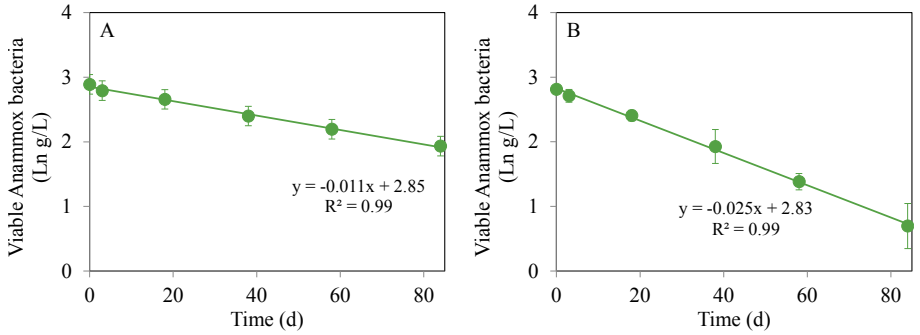

Fig. 3. Concentration of viable ananmmox bacteria during the anaerobic (A) and aerobic (DO = 1.5 mg/L) (B) starvation periods. Solid lines represent the fit of the viable Anammox bacteria concentration to the natural logarithmic function. Error bars indicate standard errors.

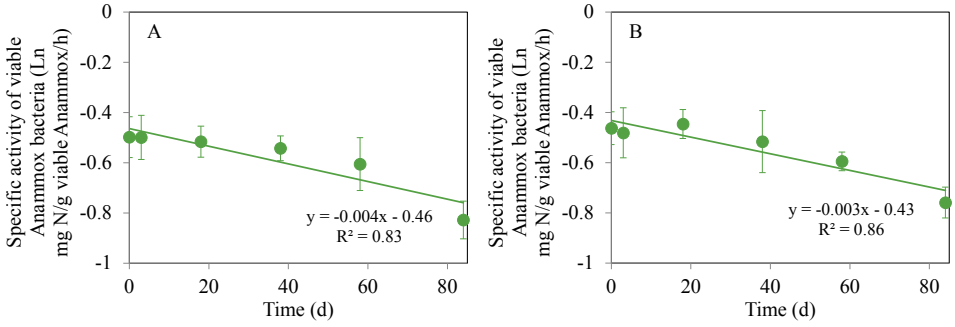

Fig. 4. Specific activity of viable Anammox bacteria during the anaerobic (A) and aerobic (DO = 1.5 mg/L) (B) starvation periods. Solid lines represent the fit of the specific activity of viable Anammox bacteria to the natural logarithmic function. Error bars indicate standard errors.

bacteria are comparable ($p > 0.05$). This trend is different from the decay and death rates of Anammox bacteria, which showed higher rates under aerobic starvation compared with anaerobic starvation. This also implied that activity decay also played a role in the decreased activity of Anammox bacteria during starvation.

3.4. Contributions of death and activity decay to the decreased Anammox activity

Based on the determined decay, death and activity decay rates of the Anammox bacteria, the contributions of death and activity decay to the decreased Anammox activities were calculated and summarized in Table 1. Table 1 shows that death contributed $73 \pm 4\%$ and $89 \pm 5\%$ to the decreased Anammox activities during the 84 days' anaerobic and aerobic starvation periods, and activity decay accounted for $27 \pm 4\%$ and $11 \pm 5\%$ of the decreased Anammox activities over the same periods. This reveals that death plays a major role in the decreased activity of Anammox bacteria, whereas activity decay is not the primary reason for the decreased Anammox activity.

In contrast to the Anammox bacteria, activity decay is mainly responsible for the decreased activities of PAOs, GAOs, heterotrophic bacteria, NOB, hydrogenotrophic and aceticlastic methanogens, as summarized in Table 2 (Hao et al., 2009, 2010a, 2012). This might be attributed to their different metabolic pathways. Heterotrophic bacteria, PAOs and GAOs are able to store large amounts of substrate as intracellular polymers like glycogen and polyhydroxyalkanoates (PHA) in the presence of external substrates (Oehmen et al., 2007; van Loosdrecht et al., 1997). Afterwards, these intracellular polymers could be consumed to produce energy for cell maintenance and/or growth over the starvation process to avoid death and/or offset dead bacteria (Oehmen et al., 2007; van Loosdrecht et al., 1997). In addition, they could also utilize the lysate of dead cells for regrowth (i.e. cryptic growth)

Table 2
Reported contributions of death and activity decay to the decreased activities of key bacteria/archaea involved in wastewater treatment (with standard errors).

Bacteria	Contribution of death (%)	Contribution of activity decay (%)	Reference
Heterotrophic bacteria	22 ± 2	78 ± 2	Hao et al. (2009)
AOB[a]	53 ± 5	47 ± 5	Hao et al. (2009)
NOB[b]	18 ± 3	82 ± 3	Hao et al. (2009)
PAOs[c]	20 ± 3	80 ± 3	Hao et al. (2010a)
GAOs[d]	26 ± 5	74 ± 5	Hao et al. (2010a)
Hydrogenotrophic methanogens	53 ± 10	47 ± 10	Hao et al. (2012)
Aceticlastic methanogens	69 ± 8	32 ± 8	Hao et al. (2012)

[a] AOB: Ammonium-Oxidizing Bacteria.
[b] NOB: Nitrite-Oxidizing Bacteria.
[c] PAOs: Polyphosphate-Accumulating Organisms.
[d] GAOs: Glycogen-Accumulating Organisms.

(Hao et al., 2010b; van Loosdrecht and Henze, 1999). NOB were reported to be facultative autotrophic bacteria and could also survive relying on the lysate of dead cells, thereby compensating dead NOB (Bock, 1976). Methanogens are able to utilize intracellular glycogen as their food during starvation (Konig et al., 1985; Roslev and King, 1995). These collectively explained why activity decay rather than death was mainly responsible for the decreased activities of PAOs, GAOs, heterotrophic bacteria, NOB and methanogens in the presence of starvation. In contrast, Anammox bacteria does not contain any PHA synthase gene and therefore was not able to synthetize PHA to produce energy under the starvation conditions (van Niftrik et al., 2008). They were not able to use glycogen as energy sources either. Consequently, starvation stress would probably kill the Anammox bacteria instead of only decreasing their specific activity, which led to a larger contribution of death to the decreased Anammox activity. This is similar to AOB, the death of which was also primarily responsible for the decreased activity compared with their activity decay (53% versus 47%) (Hao et al., 2009). This might be due to the fact that AOB were not able to synthesize PHA and could not utilize the lysate of dead bacteria either. However, the exact regulatory mechanisms of Anammox bacteria under starvation conditions are still unclear and remain to be elucidated. Therefore, in-depth studies are still needed.

3.5. Significance of this work

The decreased activity of Anammox bacteria during starvation is due to both death and activity decay, which are fundamentally different. In a word, the activity of Anammox bacteria would never get recovered following death, whereas activity decay means the Anammox bacteria could be reactivated by synthesizing new enzymes and/or switching on enzymatic activity after the favourable conditions are back. However, the death and activity decay of the Anammox bacteria have never been elucidated because of the lack of a suitable approach. This study determined the death and activity decay rates of Anammox bacteria under both anaerobic and aerobic starvation conditions for the first time based on ammonium removal rate, MLVSS concentration and molecular tools (i.e. Live/Dead staining and FISH). The contributions of death and activity decay to the decreased activities of Anammox bacteria were also first identified.

The on-going paradigm shift in wastewater treatment requires mainstream autotrophic nitrogen removal through partial nitritation followed by Anammox to maximize energy recovery (Xu et al., 2015). The quantitative evaluation of death and activity decay would undoubtedly deepen our understanding of the response of Anammox bacteria to the starvation stress, which would help the optimization of the Anammox process. This study quantitatively revealed that death played a major role (>70%) in the decreased activity of Anammox bacteria and activity decay was responsible for < 30% of the decreased Anammox activity, indicating that the Anammox bacteria would tend to die instead of decreasing enzyme regulated specific activity. This suggested that the activity of Anammox bacteria would be difficult to recover after starvation stress is imposed.

Anammox bacteria have been known to possess a slow growth rate (doubling time: 10–25 d) (Lotti et al., 2015). Therefore, it would be often time-consuming to conduct experimental work for process understanding and optimization of the Anammox systems. Mathematical models are useful tools to study the Anammox processes and provide support for the understanding and optimization of the Anammox process (Dorofeev et al., 2017; Hao et al., 2002; Ni et al., 2009, 2014). Unfortunately, the decay of Anammox bacteria is assumed to result from death only in the current modelling practice, whereas the activity decay is ignored (Dorofeev et al., 2017; Hao et al., 2002; Ni et al., 2009, 2014). Consequently, this would inevitably lead to errors in model prediction, especially for the systems with a long starvation period. Unfortunately, previous studies were not able to differentiate death from activity decay because only the traditional approach such as the biomass decrease rate was employed (Xing et al., 2016a; Zhang et al., 2015). This study experimentally showed that activity decay was also responsible for the decreased activity of Anammox bacteria although death seemed to be more important. Therefore, the model structure for Anammox bacteria needs to be modified to incorporate the activity decay. This can be done by adding an enzyme-related process, where the activity decay could be described by enzyme degradation and/or enzyme inactivation in the existing Anammox bacteria. Death could still be expressed as the decrease in the amount of Anammox bacteria.

Previous studies only measured the anaerobic decay rate of Anammox bacteria (Ma et al., 2016; Xing et al., 2016a; Zhang et al., 2015), whereas the aerobic decay rate of Anammox bacteria has never been investigated. However, the Anammox bacteria could also be exposed to the aerobic starvation condition in the real world (Lackner et al., 2014). For instance, up to 1.5 mg O_2/L has been reported to be applied to the Anammox systems (Lackner et al., 2014). The determination of the aerobic decay rate would contribute to a better understanding of the performance of the Anammox systems operated at the aerobic conditions. Also, this determined aerobic decay rate could also be included in the model to more accurately simulate the performance of the Anammox systems where the DO concentration is not zero.

It should be noted that the decay, death and activity decay rates of the Anammox bacteria were measured in the batch experiments in this study, which are different from the commonly used continuous flow systems in practice. Also, the synthetic wastewater was used in this study, whereas the real systems would receive real wastewater. These might lead to different microbial composition (e.g. bacteriophages, protozoa and viruses). These would probably

result in different decay characteristics. However, this is the generally employed approach by different research groups for the time being (Carvalheria et al., 2014; Ma et al., 2016; Salem et al., 2006; Vargas et al., 2013; Xing et al., 2016a; Zhang et al., 2015). In this respect, in situ measurement of the decay characteristics of Anammox bacteria in continuous flow systems would still be required in the future.

4. Conclusions

The decreased activity (i.e. decay) of Anammox bacteria during the anaerobic and aerobic starvation conditions was experimentally assessed for the first time in terms of death and activity decay. The main conclusions are:

- The anaerobic and aerobic decay rates of Anammox bacteria were measured at0.015 ± 0.001 d^{-1} and 0.028 ± 0.001 d^{-1}, respectively. This reveals that the Anammox bacteria would lose their activity faster in the aerobic starvation condition compared with the anaerobic starvation condition.
- The anaerobic and aerobic death rates of Anammox bacteria were determined at 0.011 ± 0.001 d^{-1} and 0.025 ± 0.001 d^{-1}, respectively.
- The anaerobic and aerobic activity decay rates of Anammox bacteria were determined at 0.004 ± 0.001 d^{-1} and 0.003 ± 0.001 d^{-1}, respectively.
- The death of Anammox bacteria was mainly responsible for their decreased activity under both anaerobic and aerobic starvation conditions, whereas activity decay played a minor role in their decreased activity.

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