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Short- and long-term effects of manganese, zinc and copper ions on nitrogen removal in nitritation-anammox process

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Abstract

This study provided a deep insight into the impacts of trace elements (Mn^{2+}, Zn^{2+} and Cu^{2+}) on nitritation-anammox process. For short-term exposure, all the three elements could improve the nitrogen removal rate (NRR) and the optimal concentrations were 2.0 mg/L, 2.0 mg/L and 0.5 mg/L for Mn^{2+}, Zn^{2+} and Cu^{2+}, respectively. Accordingly, the NRR were enhanced 54.62%, 45.93% and 44.09%. The long-term experiments were carried out in lab-scale sequencing batch reactors. The surprising results showed that only Mn^{2+} addition could enhance the long-term nitritation-anammox process, and the NRR increased from 0.35±0.01 kg N/m3/d (control, no extra trace element addition) to 0.49±0.03 kg N/m3/d. Vice versa, the amendment of Zn^{2+} reduced the NRR to 0.28±0.02 kg N/m3/d, and Cu^{2+} had no significant effect on the NRR (0.36±0.01 kg N/m3/d). From the analysis of microbial community structure, it was explained by the increasing abundance of anaerobic ammonium oxidizing bacteria (AnAOB) only in Mn^{2+} treatment, whereas Zn^{2+} predominantly promoted ammonium oxidizing bacteria (AOB). Additionally, the majority of Mn^{2+} was identified inside AnAOB cells, and Zn^{2+} and Cu^{2+} were mainly located in AOB. Our results indicated the synergistic effects of trace elements on nitritation-anammox, both short-term encouraging activities of AnAOB and long-term altering microbial community structure. This work implies the importance of trace elements addition in nitritation-anammox process.

Keywords

Nitritation-anammox process; AnAOB; AOB; manganese; zinc; copper

1. Introduction

The accelerating industrialization and urbanization in recent years has resulted in the discharge of large amounts of nitrogen-rich wastewater, consequently causing many environmental problems and attracting increasing concerns in water quality protection (Zhao et al., 2015). Meanwhile, energy consumption and carbon footprint are intensively considered in the treatment of nitrogen-rich wastewater, and it becomes one of the key challenges to apply sustainable approaches in nitrogen discharge control around the world (Shi et al., 2013). Compared to the conventional biological nitrogen removal processes, the combined nitritation-anammox process in a single reactor is suggested as an energy-efficient and sustainable wastewater treatment technology for significantly decreasing oxygen and organic carbon consumption (van der Star et al., 2007; de Graaff et al., 2011). During this process, the oxygen consumption is reduced to only 37.5% due to partial conversion of NH_4^+-N to NO_2^--N, and the surplus sludge production is minimal for high nitrogen removal efficiency and low cell growth rate. In addition, it mitigates the greenhouse gas emission by consuming CO_2 as carbon source instead of organic matters (Kartal et al., 2010). Such nitritation-anammox process has shown remarkable advantages in operation (Joss et al., 2011) and is employed in over 88% of full-scale industrial application (Lackner et al., 2014; Zhang et al., 2015a).

In nitritation-anammox system, anaerobic ammonium oxidizing bacteria (anammox bacteria, AnAOB) show highly variable responses to the external environment. Therefore, it is of great interests and challenges to enhance the activities of AnAOB in nitritation-anammox system. Recently, some exciting strategies have been used to increase the activities of AnAOB and nitrogen removal rate (NRR) by external supplementary of field energy and micronutrients, such as electric technique,

magnetic technique and ultrasonic technique (Duan et al., 2011; Zhang et al., 2012; Qiao et al., 2013). Besides, trace elements can also influent the activities of AnAOB. Low concentration of Mn, Zn, Cu are all essential micronutrients and components of many enzymes and co-enzymes for AnAOB (Strous et al., 1998). As an example, manganese oxides were respired with formate as electron donor for AnAOB *Kuenenia stuttgartiensis* (Strous et al., 2006). Cu is important constituent of nitrite reductase involved in the catabolism of AnAOB (Hira et al., 2012). Zn is also the key element for the synthetic of AnAOB enzymes that include 21 ATP-dependent zinc metal-loprotease FtsH 1 and zinc-containing dehydrogenase (Strous et al., 2006). However, excessive trace elements are toxic and might inhibit AnAOB activities. Huang's work showed that 0.05 mmol/L Mn (2.8 mg/L) can improve the activities of AnAOB and nearly double the removal efficiency (Huang et al., 2014). Kimura and Isaka found limited effects of Zn on AnAOB activity at low concentration (0.1-5 mg/L) and a dramatic inhibition behavior beyond 10 mg/L (Kimura and Isaka, 2014). Zhang also suggested that low concentration of Cu (<1.0 mg/L) promotes the AnAOB activity (Zhang et al., 2015b), whereas the suppression is observed when Cu concentration ranges from 5 mg/L to 10 mg/L.

However, most studies on anammox process mainly address AnAOB. Ammonium oxidizing bacteria (AOB) are also the functional microbes in the nitritation-anammox system, and their activities have been identified as the key to the nitritation-anammox reactor stability (Joss et al., 2011). To date, the study of metal effects on nitritation-anammox process is still lacking. Moreover, certain heavy metals, such as manganese (Mn), zinc (Zn), copper (Cu), are frequently detected in nitrogen-rich wastewater, e.g., landfill leachates, swine wastewater, and steel manufacturing wastewater (Table 1). It is becoming significantly essential as the nitritation-anammox

process has been widely used for nitrogen-rich wastewater treatment.

In this study, we evaluated the impacts on short-term and long-term nitritation-anammox of copper, manganese and zinc metals. With comprehensive analysis of the NRR and microbial community structure, we found distinct nitritation-anammox behavior, attributing to both AnAOB activity encouragement and microbial community structure alteration. Putting deeper insight into the forms and distribution of trace elements in extracellular polymeric substances (EPS) and intracellular components of nitritation-anammox sludge, we identified different active sites for heavy metal interaction on AnAOB and AOB, possibly explaining the mechanisms of trace elements affecting nitritation-anammox. The findings of this study are beneficial to the industrial nitritation-anammox practices for nitrogen-rich wastewater treatment, especially pharmaceutical wastewater.

2. Methods

2.1 Inoculum and synthetic wastewater

The sludge used for short-term and long-term experiments were collected from a laboratory-scale continuous-upflow nitritation-anammox reactor (110 cm × 10 cm × 60 cm) in Beijing Jiaotong University. The reactor had operated steadily for 1 year and the average NRR of 0.8 kg $N/m^3/d$ with the hydraulic retention time of 24 h. The dissolved oxygen (DO) was 0.1 to 0.2 mg/L and the temperature was maintained at 32±1 °C. The values of the suspended solids (SS) and volatile suspended solids (VSS) of the inoculums were 7.36 g/L and 3.44 g/L, respectively. Synthetic wastewater was composed of NH_4HCO_3 as ammonium source, basic nutrients (10.0 mg/L NaH_2PO_4, 58.6 mg/L $MgSO_4•7H_2O$ and 5.7 mg/L $CaCl_2•2H_2O$) and trace elements (Van, 1996). In 1.0 L of synthetic wastewater, the 1.25 mL of trace elements was supplemented and the composition was listed in Table S1. $KHCO_3$ solution (1250 mg/L) was added to

buffer the influent pH (8.0-8.4).

2.2 Short-term batch test

Short-term batch tests were performed for 24 hours to explore the optimal concentration of trace elements to achieve the highest NRR. The tests were carried out in 250 mL serum vials containing 200 mL synthetic wastewater. Biosludge was taken from the laboratory-scale nitritation-anammox reactor and washed three times with mineral medium to remove residual nitrogen. According to (Daverey et al., 2014a), the mixed liquid suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were set as 3159 and 1458 mg/L, respectively. The initial NH_4^+-N was 150 mg/L. Then, the vials were sealed by sealing film, and the sealing film had a hole with the diameter 1 cm to make the reactor operated under a micro-aerobic condition. The vials were then placed in a thermostatic shaker, the dissolved oxygen (DO) was 0.1 to 0.2 mg/L when the speed was 150 rpm. The temperature was maintained at 32±1 °C and pH was controlled at 8.0-8.4 by adding $KHCO_3$ solution to the influent. The temperature, pH and DO were monitored by pH/oxi340i and corresponding probes (WTW, Germany). Samples were obtained every three hours using a syringe needle to analyze the concentrations of NH_4^+-N, NO_2^--N and NO_3^--N. The amendment of extra trace elements ranged from 0 to 20 mg/L for Mn^{2+} (in terms of $MnCl_2 \cdot 7H_2O$) and Zn^{2+} ($ZnSO_4 \cdot 7H_2O$), and from 0 to 10 mg/L for Cu^{2+} ($CuSO_4 \cdot 5H_2O$).

2.3 Long-term continuous test

Four reactors were operated for 90 days to examine the long-term effects of trace elements on nitritation-anammox process. Four identical sequencing batch reactors had the working volumes of 0.5 L with the inner diameter of 5 cm and the height of 25.5 cm. The four reactors included Control (no extra trace element addition), Mn treatment, Zn treatment and Cu treatment. In Control, the addition of trace elements

followed the same as short-term test, and the trace elements in other reactors followed the optimal concentration obtained in short-term experiment (2.0 mg/L for Mn^{2+} ($MnCl_2 \cdot 7H_2O$), 2.0 mg/L for Zn^{2+} ($ZnSO_4 \cdot 7H_2O$) and 0.5 mg/L for Cu^{2+} ($CuSO_4 \cdot 5H_2O$)). The initial SS and VSS concentrations were 3082 and 1326 mg/L, respectively. DO was controlled at 0.1-0.2 mg/L by changing the aeration rate and the temperature was maintained at 32±1 °C. $KHCO_3$ solution was added to buffer the influent pH (8.0-8.4). The temperature, pH and DO were monitored by pH/oxi340i and corresponding probes (WTW, Germany). The hydraulic retention time was 24 h and the volume exchange ratio was 0.5. The solid retention time (SRT) was mainly 10 d. The long-term operation was divided into two phases (Table 2).

2.4 Calculations

2.4.1. Calculation of NRR

$$\mathrm{NRR\ (kg\ N/m^3/d)} = \frac{\mathrm{NH_4^+-N(inf-eff)+NO_2^--N(inf-eff)+NO_3^--N(inf-eff)}}{\mathrm{t\times 1000}} \quad (1)$$

The NH_4^+-N_{inf}, NH_4^+-N_{eff}, NO_2^--N_{inf}, NO_2^--N_{eff}, NO_3^--N_{inf} and NO_3^--N_{eff} were the NH_4^+-N, NO_2^--N and NO_3^--N concentration in the influent and effluent during long-term operation, respectively; t is the hydraulic retention time, (d).

2.4.2. Calculation of nitrogen transformation of AOB and AnAOB

AOB and AnAOB are the main functional microorganisms for the nitrogen biotransformation in the reactors. Part of ammonium is oxidized to nitrite by AOB (Eq. 2). Then, the remaining ammonium and nitrite are converted to nitrogen gas by AnAOB (Eq. 3) (Miao et al., 2016). The ammonium conversion rate (ACR) by AOB (AOB-ACR) (Eq. 4) and AnAOB (AnAOB-ACR) (Eq. 5) are estimated to explain the activity of AOB and AnAOB.

$$NH_3+1.5O_2 \rightarrow NO_2^-+H_2O+H^+ \quad (2)$$

$$NH_4^++1.32NO_2^-+H^+ \rightarrow 1.02N_2+0.26NO_3^-+2H_2O \quad (3)$$

$$AOB-ACR=\frac{NH_4^+-N(inf-eff)-\frac{NH_4^+-N(inf-eff)-NO_2^--N(eff-inf)-NO_3^--N(eff-inf)}{2.06}}{t\times1000} \quad (4)$$

$$AnAOB-ACR=\frac{NH_4^+-N(inf-eff)-NO_2^--N(eff-inf)-NO_3^--N(eff-inf)}{2.06\times t\times1000} \quad (5)$$

The NH_4^+-N_{inf}, NH_4^+-N_{eff}, NO_2^--N_{inf}, NO_2^--N_{eff}, NO_3^--N_{inf} and NO_3^--N_{eff} were the NH_4^+-N, NO_2^--N and NO_3^--N concentration in the influent and effluent during long-term operation, respectively; t is the hydraulic retention time, (d). It was assumed that the nitrogen assimilation due to heterotrophic growth was neglected in the calculations.

2.5 Microbial community structure analysis

Biosludge was collected from the four treatments at the beginning (0 day) and end (90 day) of long-term continuous test. The samples were centrifuged at 10,000 rpm for 10 min and the DNA of biomass pellets was extracted. The ammonia monooxygenase encoding genes of AOB (*amoA*) and AnAOB were amplified using the primer sets amoA-1f/amoA-2r and Amx368f/Amx820r, respectively (Table S2). Quantitative real-time polymerase chain reaction (qPCR) was used to amplify the targeting ammonia monooxygenase encoding genes and the universal bacterial 16S rRNA using the SYBR Green qPCR Kit (Liu et al., 2012). The amplification efficiencies were between 97.63% and 105.82% following the slopes of calibration curve (Table S3).

2.6 EPS extraction

A heat-extraction method was employed for extracellular polymeric substances (EPS) extraction (Yin et al., 2015a), details in Supplementary Materials. Polysaccharide measurement was acquired using the anthrone method with a glucose standard, and protein levels were measured using the modified Lowry method with bovine serum albumin as a standard (Wu et al., 2009).

2.7 Chemical analysis

The trace elements in biosludge were categorized into soluble fraction, EPS-absorbed fraction and intracellular fraction. The water samples were taken from each reactor at the beginning and end of each test. The soluble metal concentrations in the supernatant were filtered by 0.45 µm acetate cellulose membranes before analysis. Then AnAOB and AOB were separated by modified differential centrifugation method (DC) (Boelee et al., 2014)(supporting material). For intracellular metals, the 0.1 g (wet weight) of biomass washed with a modified ethylenediaminetetraacetic acid (EDTA) washing procedure to remove the soluble and absorbed metal ions (And and Wilkinson, 2000; Vasconcelos and Leal, 2001; Hu et al., 2003). The pellets were dissolved by nitric acid solution and then centrifuged to obtain supernatant (Bi et al., 2014). The EPS-absorbed metals were calculated from the difference between the total and the measured soluble/intracellular metal concentrations (Hu et al., 2003). The concentrations of trace elements were detected by inductively coupled plasmaoptical emission spectrometry (ICP-OES) (Perkin Elmer Optima 8300DV).

The temperature, pH and DO were monitored by pH/oxi340i and corresponding probes (WTW, Germany). For other chemicals, the water samples were centrifuged at 3000 rpm for 1 min. The supernatants were filtered by 0.45 µm acetate cellulose membranes, followed by standard methods for the analysis of ammonium, nitrite, nitrate, SS, VSS, MLSS and MLVSS (APHA, 2005).

2.8 Data analysis

All samples were performed in triplicates, and the results were expressed as the mean ± standard deviation. An analysis of variance (ANOVA) was used to test the significance of the results, and $p< 0.05$ was considered to be statistically significant. A statistical comparison between variables was performed using the t-test for a normally

distributed dataset by SPSS Version 18.

3. Results and discussion

3.1 Short-term effects of trace elements on nitritation-anammox process

The results of short-term exposure suggested that appropriate amendment of Mn^{2+}, Zn^{2+} and Cu^{2+} enhanced the performance of nitritation-anammox process by increasing the NRR, as illustrated in Fig. 1. The NRR with different Mn^{2+} amendment followed the bell shape, increasing from 0.060 kg $N/m^3/d$ (0 mg/L) to the peak of 0.093 kg $N/m^3/d$ (2.0 mg/L, 54.62% higher), and then declining to 0.021 kg $N/m^3/d$ (20 mg/L). The residual NO_2^--N at 24 hours was found when Mn^{2+} concentration was above 5.0 mg/L, implying the strong inhibition of nitritation-anammox process by the excessive Mn^{2+}. Similarly, the bell shapes of Zn^{2+} and Cu^{2+} also indicated that the optimal amendment of these two trace elements was 2.0 mg/L and 0.5 mg/L, and the according NRR was 0.088 kg $N/m^3/d$ and 0.087 kg $N/m^3/d$, respectively. The significant accumulation of NO_2^--N at 24 hours was observed when Zn^{2+} concentration was above 3.0 mg/L and Cu^{2+} concentration was over 2.0 mg/L. The results suggested that appropriate addition of trace elements could significantly improve the performance of nitritation-anammox process and the optimal amendment was 2.0 mg/L for Mn^{2+} and Zn^{2+}, and 0.5 mg/L for Cu^{2+}.

3.2 Long-term effects of trace elements on nitritation-anammox process

The nitrogen removal dynamics in the four treatments were illustrated in Fig. 2 and Fig. S1, and there were significant differences in nitrogen removal performance between treatments. Without exposure to the excessive trace elements in Control (Fig. 2A), the average NRR increased from 0.14±0.01 to 0.34±0.01 kg $N/m^3/d$. The ratio of ammonium conversion concentration by AOB to ammonium conversion concentration by AnAOB implied the activity of AOB and AnAOB, and the

theoretical value was 1:1. The higher of the ratio, the higher activity of AOB. In phase I, the ratio increased from 1.25±0.04 to 1.30±0.02. In Phase II, the NRR did not increase, consequently causing the excessive NH_4^+-N in effluent when the influent NH_4^+-N concentration was above 400 mg/L. And the ratio increased to 1.38±0.02 from Day 61-71.

In Mn treatment, the effluent NO_3^--N had a slight increase from 11.54 to 39.15 mg/L (the ratio was mainly 1.51±0.20, Fig. S1) during the initial 7 days and then sharply dropped. It might be explained by NO_3^--N reduction to N_2 by the reaction between Mn^{2+} and NO_3^--N (Luther et al., 1997). The NRR increased slightly from 0.34±0.01 to 0.37±0.01 kg N/m^3/d from day 47 to 61. Subsequently in Phase II, the NRR continuously increased and reached the maximum value of 0.49±0.03 kg N/m^3/d, much higher than that of Control. Furthermore, the effluent NH_4^+-N, NO_2^--N and NO_3^--N remained at very low concentrations. The results suggested that the additive of 2.0 mg/L Mn^{2+} significantly improved the NRR of nitritation-anammox process. Similar phenomenon has been reported previously. Huang found that the maximum NRR was 1.97 kg N/m^3/d in long-term anammox process when Mn^{2+} concentration was 2.8 mg/L (Huang et al., 2014). The positive effect of MnO_2 on anammox process was also proved by the 2-folds higher NRR than that without MnO_2 addition (Qiao et al., 2012).

Different from the NRR promotion in short-term tests, 2.0 mg/L Zn^{2+} suppressed the anammox process in long-term experiment. In Phase I, the NRR (0.13±0.01 kg N/m^3/d to 0.22±0.01 kg N/m^3/d) was basically similar to Control (0.14±0.01 kg N/m^3/d to 0.23±0.01 kg N/m^3/d) during the Day 1-30. Then, NRR gradually increased to 0.28±0.02 kg N/m^3/d from Day 31-61 which was lower than Control (0.34±0.01 kg N/m^3/d). The effluent concentrations of NH_4^+-N, NO_2^--N and NO_3^--N were therefore

relatively higher. Particularly in Phase II when influent NH_4^+-N was above 400 mg/L, the NRR declined to 0.22±0.02 kg N/m^3/d. Our results did not fit with previous researches, suggesting a different mechanism of the impacts of Zn^{2+} on anammox process. Zn^{2+} was reported to stimulate the NRR of the simultaneous partial nitrification, anammox and denitrification (SNAD) process when the concentration was below 10 mg/L (Daverey et al., 2014b). Meanwhile, the NRR of anammox process was not affected by 2.0 mg/L Zn^{2+} in influent (Kimura and Isaka, 2014). It might be explained by the unique microbial community composition and microbial growth rates (Wang et al., 2010; Qiao et al., 2013), or the different characteristics in various anammox processes (e.g., anammox process, nitritation-anammox process and SNAD process). As a consequence, a higher concentration of NO_2^--N was observed in Zn treatment (Fig. 2C), and it inhibited the acitivites of AnAOB and decreased the NRR.

The trends of effluent nitrogen species and NRR in Cu treatment were similar to Control without signicant difference (p=0.696). The results indicated that 0.5 mg/L Cu^{2+} had no effect on nitritation-anammox process. Similarly, the anammox activity was not influenced by 0.06-2.0 mg/L Cu^{2+} in influent from Kimura's continuous feeding tests (Kimura and Isaka, 2014). Low levels of Cu (4.0 mg/L) did no inhibit the anammox activity (Yang et al., 2013).

Besides, AOB-ACR and AnAOB-ACR were calculated to reveal the response of AOB and AnAOB to metals (Fig. 3). In Mn treatment, AOB-ACR increased from 0.08 to 0.33 and AnAOB-ACR increased from 0.07 kg N/m^3/d to 0.28 kg N/m^3/d in Mn treatment, both significantly higher than those in Control. The results fitted well with NRR results and further proved the positive promotion of Mn^{2+} on the activities of AOB and AnAOB. Similarly, AnAOB-ACR and AOB-ACR increased to 0.15 kg

293 $N/m^3/d$ and 0.25 kg $N/m^3/d$ in Zn treatment, respectively. The average AOB-ACR was
294 similar to that (0.23 kg $N/m^3/d$) in Control, whereas AnAOB-ACR was much lower
295 compared to Control reactor (0.19 kg $N/m^3/d$). The results hinted that Zn^{2+} did not
296 affect the activity of AOB but strongly inhibited AnAOB. It might be explained by the
297 specific binding of Zn^{2+} to the active sites of ammonia monooxygenase in AOB and
298 positive contribution to their growth and metabolic activities (Gilch et al., 2009; Lee
299 et al., 2011). As a consequence, a higher concentration of NO_2^--N was observed in Zn
300 treatment (Fig. 2C), and it inhibited the acitivites of AnAOB and decreased the NRR.
301 In Cu treatment, there was no significant change in AOB-ACR and AnAOB-ACR.

302 **3.3 Alterations of microbial community structure**

303 To further investigate the microbial community change in long-term
304 nitritation-anammox process, the abundance of AOB, AnAOB, nitrite oxidizing
305 bacteria(NOB) and denitrifying bacteria was evaluated by qPCR at day 1 and day 90,
306 as illustrated in Fig. 4 and Table S4. As the limited DO and none organic material,
307 NOB and denitrifying bacteria were all below the limit of detection and these bacteria
308 were ignored in this study. At day 1, the 16S rRNA copy numbers of total bacteria
309 were $(1.14\pm0.01) \times 10^7$ copies/mg SS. AOB and AnAOB accounted for 31.80%-34.95%
310 and 2.41%-2.51% of the total population, respectively. After 90 days experiments, the
311 abundance of total bacteria maintained similarly, ranging from 1.08×10^8 to $4.33 \times$
312 10^8 copies/mg SS. In Control, the abundance of AnAOB remained stable (2.68%),
313 whereas a significant increasing abundance of AOB was observed (42.74%). In Cu
314 treatment, the abundance of AOB and AnAOB remained similarly as Control, 46.81%
315 and 2.72%, respectively. In Mn treatment, the abundance of AnAOB dramatically
316 increased to 4.50% and AOB abundance was similar to that of Control with no
317 significant difference (p=0.179). In contrast, a remarkable increase of AOB

abundance from 42.74% to 80.66% was observed in Zn treatment, while AnAOB abundance slightly decreased to 2.46%.

From the different impacts of trace elements on the nitrogen removal performance in both short-term and long-term experiments, we found two mechanisms explaining the phenomenon. Firstly, the appropriate addition of trace elements (Mn^{2+}, Zn^{2+} or Cu^{2+}) in short-term test can significantly encourage the activities of AnAOB, which are well-known as the restriction factor in nitritation-anammox system. Secondly, the composition of microbial community determines the nitrogen removal functions of nitritation-anammox sludge. To gain a quantitative insight into the relative contributions of these factors to NRR, three equations were built, as shown in Fig. 5. The positive correlation of NRR with AnAOB-ACR and AnAOB abundance were observed, with R^2 values over 0.9. The results provided evidence that the activities of AnAOB and abundance of AnAOB were related to NRR. Wang et al. (2016) also testified this phenomenon. It is also evidenced that AnAOB is the key microbes in nitritation-anammox process to remove nitrogen. In this study, Mn^{2+} not only promoted the short-term activities of AnAOB (Fig. 1) but also enhanced their long-term abundance (Fig. 4), resulting in the significant increase of the NRR. Though Zn^{2+} increased the activities of AnAOB and the total population of the sludge from $(1.14\pm0.10) \times 10^8$ to $(4.33\pm0.32) \times 10^8$ copies/mg SS, the relative abundance of AnAOB decreased due to the faster growth rate of AOB. Many previous researches demonstrated that the presence of metals could influence microbial abundance in wastewater treatment systems (Stasinakis et al., 2002; Kelly et al., 2004; Qiao et al., 2013). Copper was reported to influence the microbial populations and NH_4^+-N removal rates in wastewater biological treatment (Sun et al., 2016). For the first time, we find the distinct two mechanisms of trace elements can simultaneously influence

long-term nitritation-anammox by promoting AnAOB activities and altering microbial community structure.

3.4 The change of EPS in AOB and AnAOB

Contents and compositions of EPS are related with their functions in reactors, and proteins (PN) and polysaccharide (PS) are the restriction components (Hou et al., 2015). The contents of PN and PS in the EPS extracted from nitritation-anammox sludge were shown in Table 3. At day 1, the EPS of nitritation-anammox sludge was 133.89±8.47 mg/g SS. PN and PS contents were 76.61±7.00 mg/g SS and 57.28±4.77 mg/g SS, respectively. The ratio of PN to PS ranged from 1.31 to 1.35. After 90 days experiments, the EPS had a slightly increase in Control, Mn and Cu treatments to 140.61±2.73 mg/g SS. The PN/PS ratio did not significantly change in Control (1.33) and Cu (1.36) treatment, whereas it remarkably decreased to 1.23 in Mn treatment due to a slight increasing content of PS (62.11±2.61 mg/g SS). In contrast, the contents of PN and PS in Zn treatment dramatically increased to 93.57±3.14 mg/g SS and 69.07±2.64 mg/g SS, respectively. And a remarkable increase of EPS from 135.22±4.39 to 162.64±4.10 mg/g SS was observed. Nevertheless, the PN/PS ratio had no significant change (1.35). The results were different from previous study which revealed the distinct PN/PS ratio in AnAOB-enriched (2.64±0.12) and AOB-enriched (0.56±0.03) sludge (Yin et al., 2015b). In the present study, the nitritation-anammox sludge was composed of both AnAOB and AOB, and the EPS came from both bacteria and were of average values. Meanwhile, the increasing EPS in Zn treatment might be explained by the higher EPS contents in nitritaion-anammox sludge which enhance the neighboring microbial cells adhesion to cope Zn inhibition (Zhang et al., 2015c). Additionally, the PN/PS ratio has a strong correlation with sludge settleability (Basuvaraj et al., 2015). The decreasing PN/PS ratio in Mn

368 treatment suggested that Mn could enhance the settleability of nitritation-anammox
369 sludge.

370 **3.5 Mechanisms of metals promoting AOB and AnAOB**

371 The fractions and distribution of trace elements in the nitritation-anammox sludge
372 were shown in Fig. 6 and Table 4. In the nitritation-anammox process, metal ions
373 were added as the EDTA coordination compounds (Strous et al., 1998). The
374 metal-chelator could enhance the solubility and bio-availability of metal ions, and
375 promote the absorption and utilization by anaerobic microorganism (Vintiloiu et al.,
376 2013). Even though EDTA could enhance the solubility and bio-availability, the
377 precipitation reactions would occurred when the total metal ions concentrations
378 reached high level. Li et al. (2015) also demonstrated that the precipitate reactions can
379 reduce the soluble levels of Cu^{2+} and Zn^{2+} when the concentrations were above 2
380 mg/L. And this part of metal precipitates could hardly be utilized by anaerobic
381 microorganism. In this study, the metal precipitated were included in EPS-absorbed
382 fractions.

383 At day 1, the concentrations of the three metals were below the limit of detection.
384 After 90 days' long-term experiment, the soluble metals remained below the limit of
385 detection, whereas the EPS-absorbed and intracellular fractions both increased. The
386 results indicated that all the metals were absorbed by biosludge. In control, Mn^{2+}
387 bound to AnAOB-EPS and AnAOB-associated were 0.38±0.02 mg/g SS (17.27% of
388 total Mn^{2+}) and 1.01±0.17 mg/g SS (45.91%). And Mn^{2+} in AOB-EPS and
389 AOB-associated were 0.32±0.01 mg/g SS (14.55%) and 0.49±0.13 mg/g SS (22.27%).
390 In Mn treatment, Mn^{2+} found in intracellular fraction of AnAOB was higher than
391 control (6.69±0.76 mg/g SS, 51.34%), followed by intracellular fraction in AOB
392 (2.43±0.45 mg/g SS, 18.65%). It suggested that the Mn^{2+} was predominantly

absorbed and uptaken by AnAOB, explaining the positive roles of Mn^{2+} in promoting enzymatic activities of AnAOB and the NRR in nitritation-anammox process.

The dominant Zn^{2+} was intracellular fraction in AOB (4.97±0.49 mg/g SS, 40.64% of total Zn^{2+}) in Zn treatment which was significantly higher than control (19.11%). The results were consistent with the study by Gilch and Lee (Gilch et al., 2009; Lee et al., 2011). Meanwhile, the major proportion of Zn^{2+} in AnAOB was identified as EPS-absorbed fraction (4.55±0.24 mg/g SS, 37.20%), similar as the previous conclusion from Daverey's work (Daverey et al., 2014b). The results hinted that, though Zn could encourage AnAOB activities, it was favorably uptaken by AOB cell and its contribution to AnAOB was limited. Thus, AnAOB were not significantly promoted by the additive Zn in long-term nitritation-anammox process to enhance the NRR.

In Cu treatment, the distribution of Cu^{2+} was similar to Control. The intracellular Cu^{2+} was the major component in the sludge, 3.97±0.29 mg/g SS (46.76% of total Cu^{2+}) for AOB and 2.87±0.15 mg/g SS (33.80%) for AnAOB. The results showed that Cu^{2+} was mainly located within the microbial cells, more in AOB than AnAOB to explain the limited contribution of Cu addition to NRR improvement. Due to the multiple-layer structure in anammox granules (Zhang et al., 2015c), Cu^{2+} could be rapidly internalized and attained equilibrium within 4 hours in floating sludge (Hu et al., 2003). It therefore helped our understanding why Cu^{2+} in anammox reactor was almost evenly dispersed, similar to previous study (Zhang et al., 2015c).

4. Conclusions

In the present study, we revealed the distinct impacts of trace elements on nitritation-anammox process for the first time. Though the short-term addition of trace elements can encourage the activities of AnAOB and improve nitrogen removal

efficiency, we found the second mechanisms that long-term exposure to trace elements alters the microbial community structure of anammox sludge. From deeper insight into the slurry EPS and metal distribution, this phenomenon was attributed to the different biosorption and uptake of trace elements between AOB and AnAOB. Due to such synergistic effects, trace elements are important and complex factors affecting nitrogen removal performance and should be carefully detected, when employing nitritation-anammox process in nitrogen-rich wastewater treatment and adding nutrients to promote anammox performance. The conclusions of this research help our better understanding how to manage and enhance nitrogen removal performance in practical nitritation-anammox process.

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Figure Captions

Fig. 1. Nitritation-anammox NRR after short-term exposure to trace elements. (A) Mn treatment; (B) Zn treatment; (C) Cu treatment.

Fig. 2. Nitrogen removal rate (NRR) and nitrogen species variation after long-term exposure to trace elements. (A) Control; (B) Mn treatment; (C) Zn treatment; (D) Cu treatment.

Fig. 3. Ammonia conversation rate (ACR) by AOB (AOB-ACR) and AnAOB (AnAOB-ACR). (A) Control; (B) Mn treatment; (C) Zn treatment; (D) Cu treatment.

Fig. 4. Abundance of anammox bacteria (AnAOB) and ammonium oxidizing bacteria (AOB) before and after long-term exposure to trace elements.

Fig. 5. Correlation between AnAOB abundance, NRR, AOB-ACR and AnAOB-ACR in different trace element treatments.

Fig. 6. Distribution of trace elements in bacterial EPS or intracellular components before and after long-term exposure to trace elements. (A) Mn in control and Mn treatment reactor; (B) Zn in control and Zn treatment reactor; (C) Cu in control and Cu treatment reactor.

602 # Tables

603 **Table 1.** Concentrations of zinc in different nitrogen rich wastewater streams.

wastewater	Manganese concentration(mg/L)	Zinc concentration (mg/L)	Copper concentration (mg/L)	NH_4^+-N concentration (mg/L)	References
Pharmaceutical wastewater	0.01-3.5	0.05-18.01	0-33.18	45.1-3580	This study[a]
Rare earth wastewater	0.8-12.4	0.6-1.92	0.037-1.14	43-4081	
Vitamin B12 production wastewater	0.046-8.37	2.67-73.11	4.69-387.63	26.5-1046.6	
Landfill leachate	0.03-1400	0.03-1000	0.005-10	50-2200	(Peter et al., 2002)
Swine wastewater		0.25-26.3	0.36-26.8	11-872	(Vanotti et al., 2007)
Mine water	40.1-65.7	20-27	1050-1550		(Stankovic et al., 2009)
Steel manufacturing	0.5-2100		0.002-0.03	50-600	(Lydon, 2000)

604 [a]: the four kinds of wastewater in this study are sampled from the production factories and detected by ICP-MS.

605 **Table 2. The conditions of the nitritation-anammox process during the two phases.**

Phase	Operation period (d)	Nitrogen loading rate (kg $N/m^3/d$)	pH	DO
Phase I	1-7	0.15±0.004	8.0-8.4	0.1-0.2
	8-17	0.22±0.004	8.0-8.4	0.1-0.2
	18-30	0.27±0.005	8.0-8.4	0.1-0.2
	31-46	0.32±0.006	8.0-8.4	0.1-0.2
	47-61	0.40±0.006	8.0-8.4	0.1-0.2
Phase II	62-71	0.05±0.005	8.0-8.4	0.1-0.2
	72-90	0.04±0.007	8.0-8.4	0.1-0.2

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609 **Table 3.** The change of EPS components before and after long-term exposure to trace elements.

Treatment	Time (d)	Protein (mg/g SS)	Polysaccharide (mg/g SS)	EPS (mg/g SS)	PN/PS
Control	1	76.48±2.32	56.76±1.82	133.24±2.95	1.35
	90	79.48±4.64	59.70±1.89	139.18±5.01	1.33
Mn	1	76.01±3.85	56.65±2.65	132.67±4.68	1.34
	90	76.67±3.13	62.11±2.61	138.77±4.07	1.23
Zn	1	76.66±3.72	58.56±2.32	135.22±4.39	1.31
	90	93.57±3.14	69.07±2.64	162.64±4.10	1.35
Cu	1	77.27±2.96	57.16±2.21	134.43±3.70	1.35
	90	81.14±2.31	59.47±1.46	140.61±2.73	1.36

610 Note: PN and PS refer to the protein and polysaccharide, respectively.

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615 **Table 4.** The concentrations of trace elements in bacterial EPS or intracellular components after long-term exposure to trace elements.

	AOB-associated (mg/g SS)	AnAOB-assocaited (mg/g SS)	AOB-EPS (mg/g SS)	AnAOB-EPS (mg/g SS)
The concentration of Mn^{2+} in R0(Mn in control) and R1(Mn treatment reactor)				
R0	0.49±0.13	1.01±0.17	0.32±0.01	0.38±0.02
R1	2.43±0.45	6.69±0.76	1.65±0.27	2.26±0.36
The concentration of Zn^{2+} in R0(Zn in control) and R2(Zn treatment reactor)				
R0	0.43±0.06	0.52±0.03	0.37±0.08	0.93±0.07
R2	4.97±0.49	1.05±0.27	1.66±0.36	4.55±0.24
The concentration of Mn^{2+} in R0(Cu in control) and R3(Cu treatment reactor)				
R0	1.47±0.13	0.74±0.05	0.24±0.02	0.56±0.02
R3	3.97±0.29	2.87±0.15	0.53±0.13	1.12±0.11

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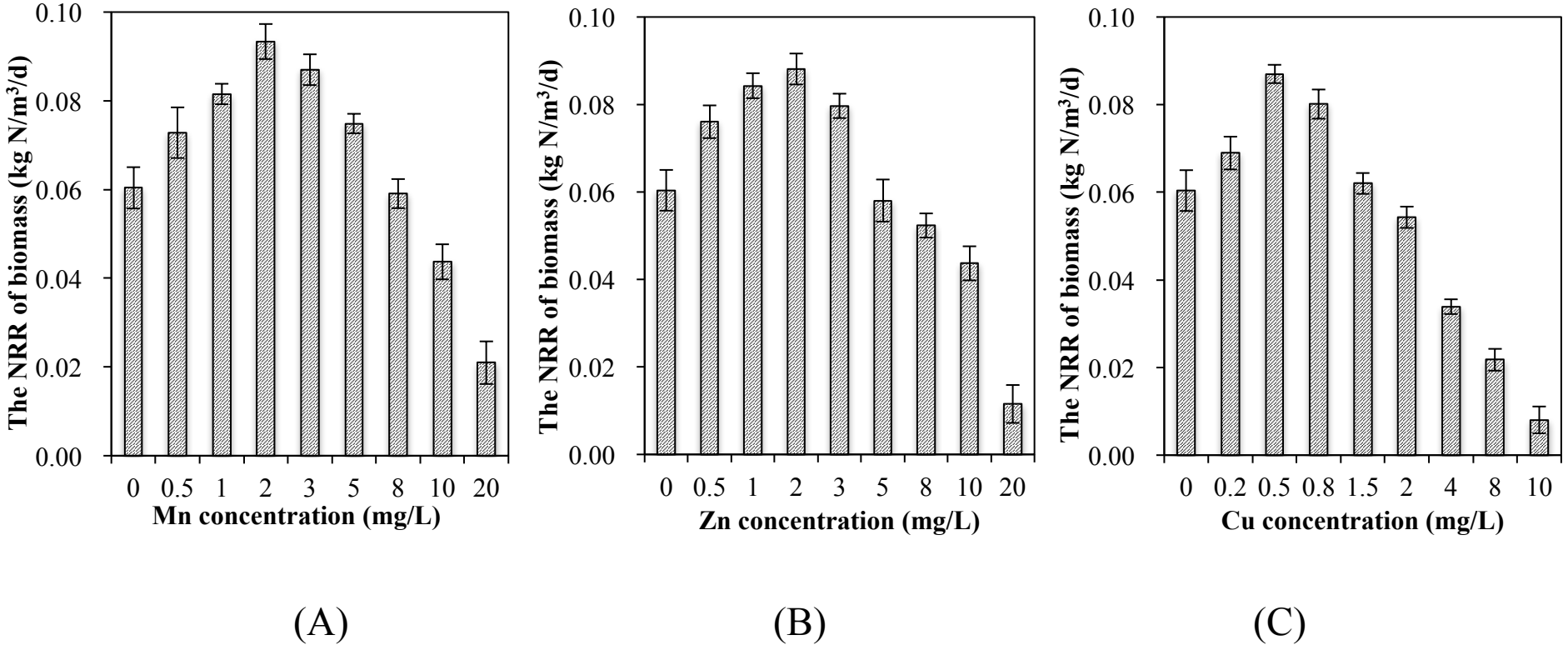

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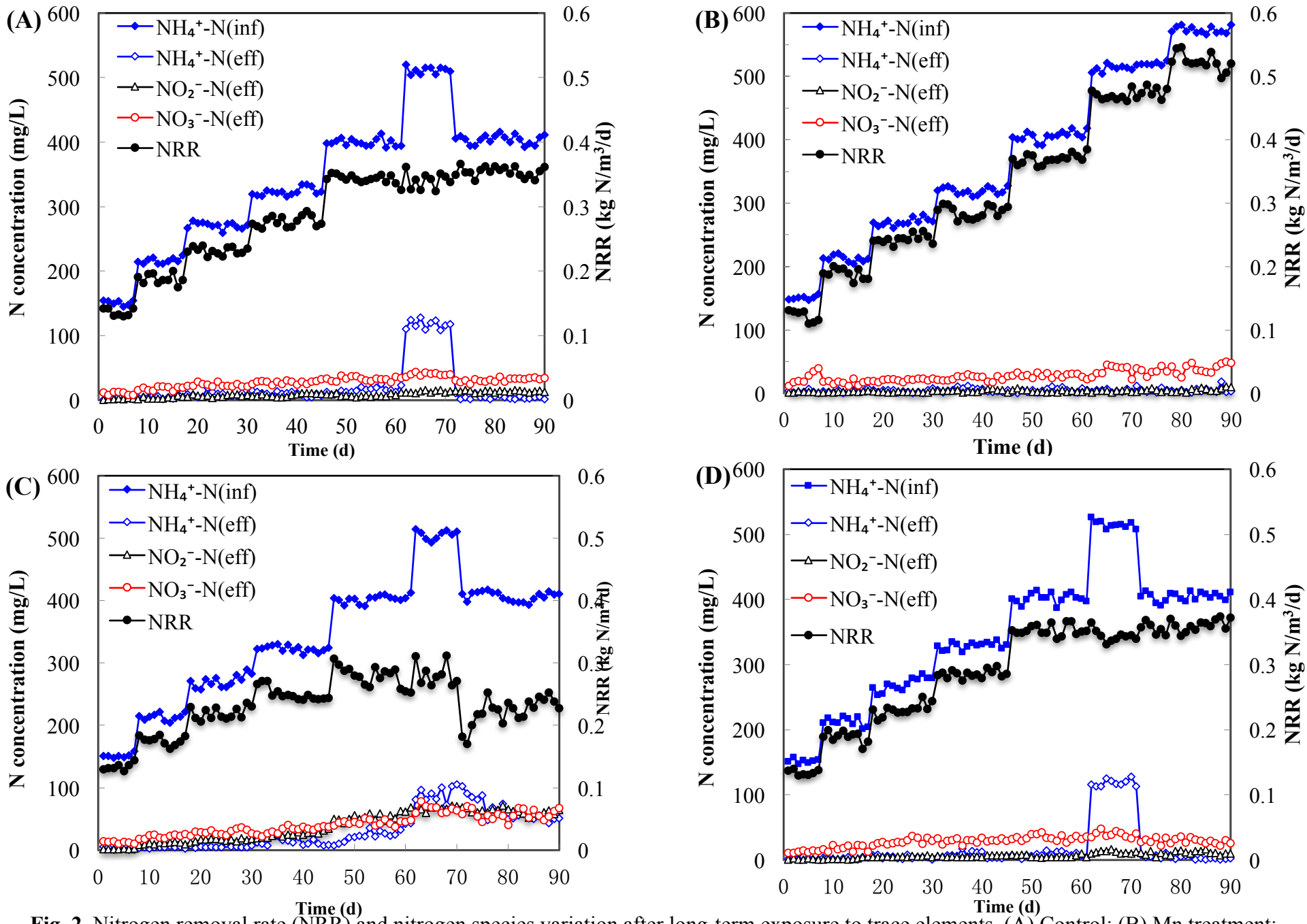

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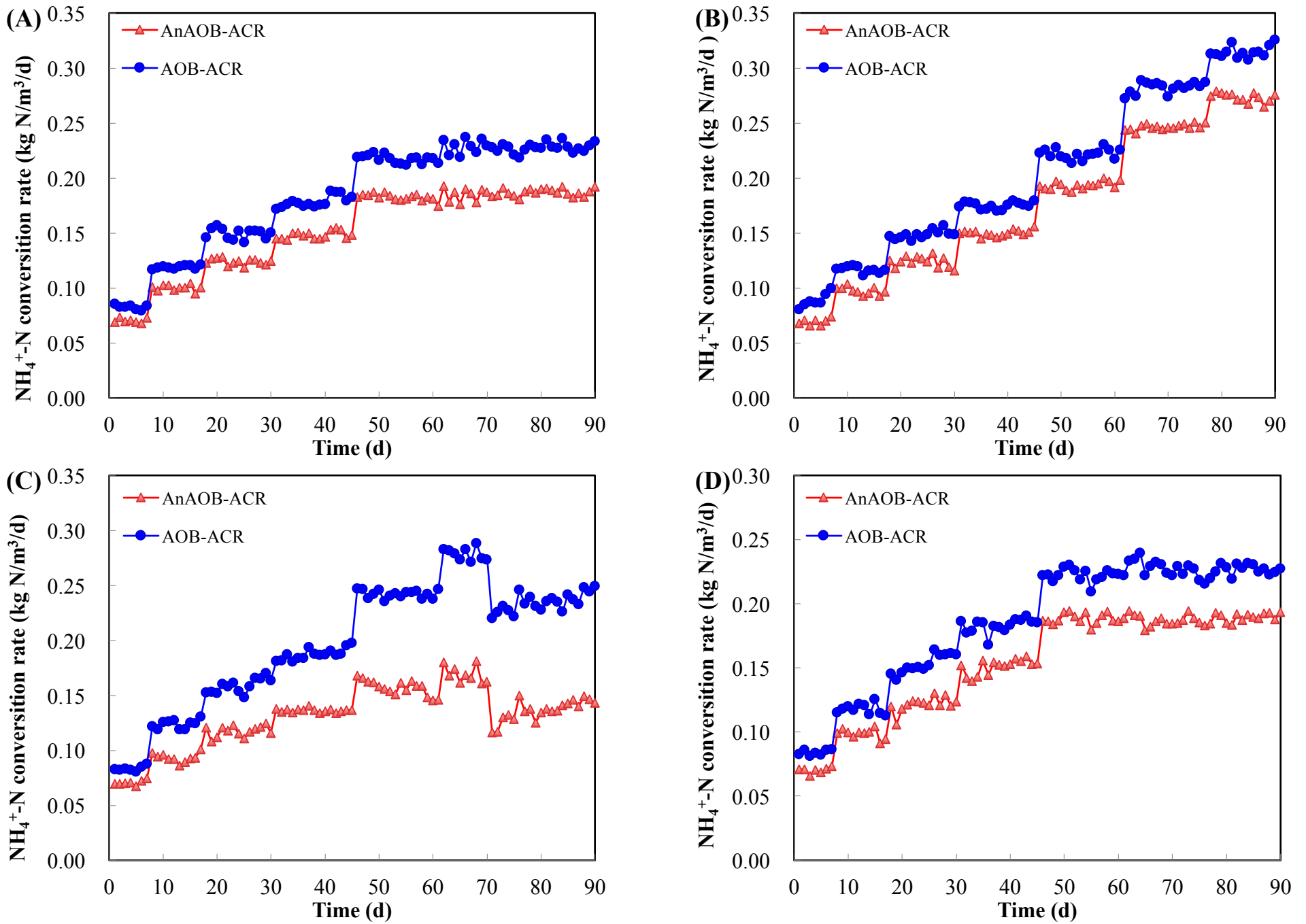

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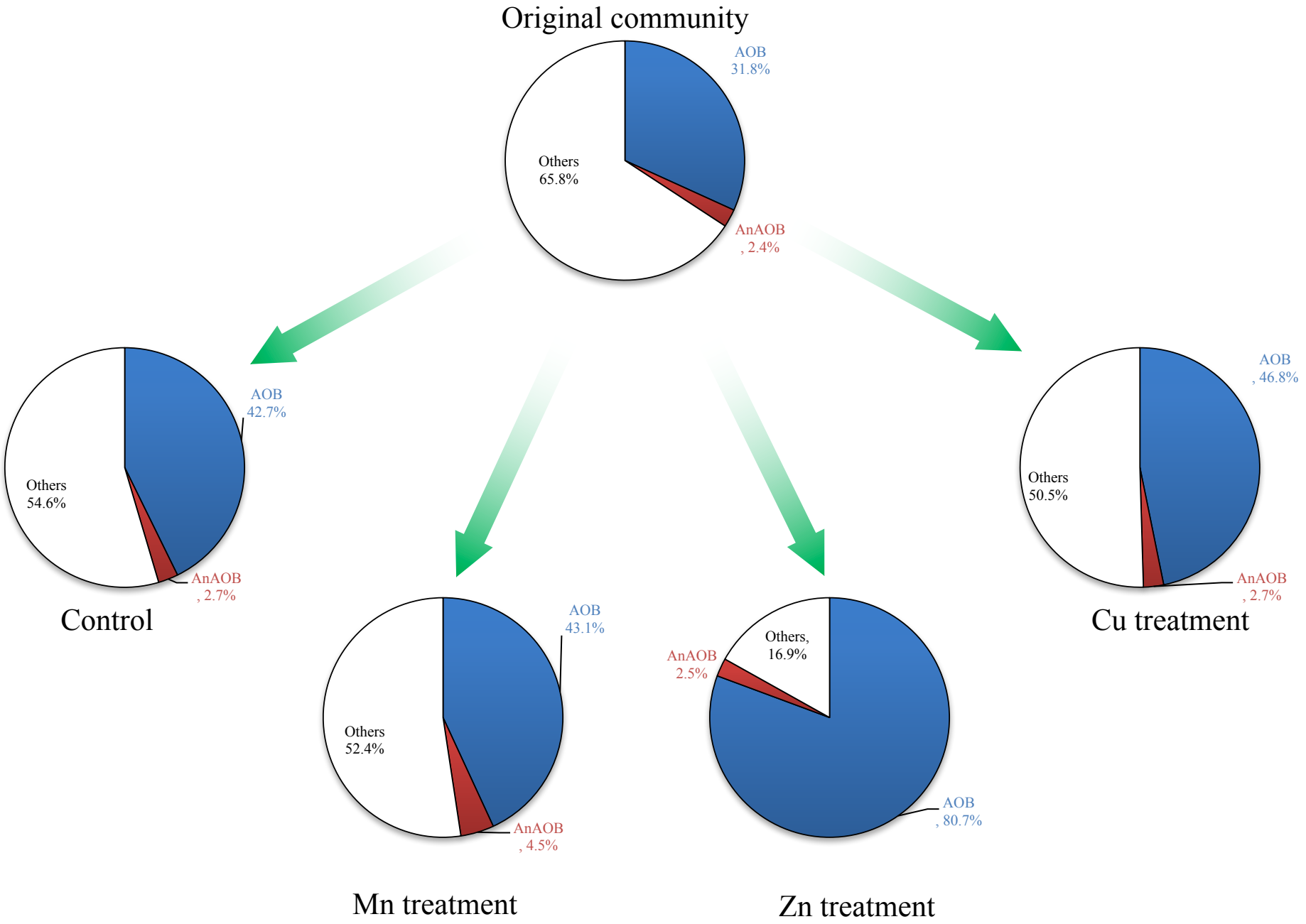

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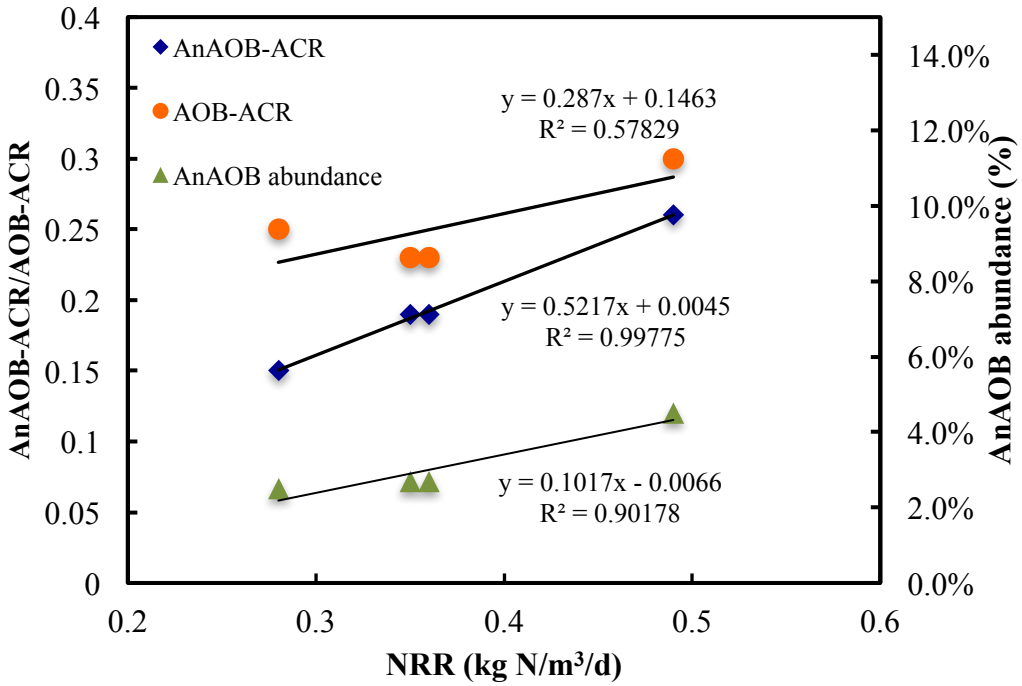

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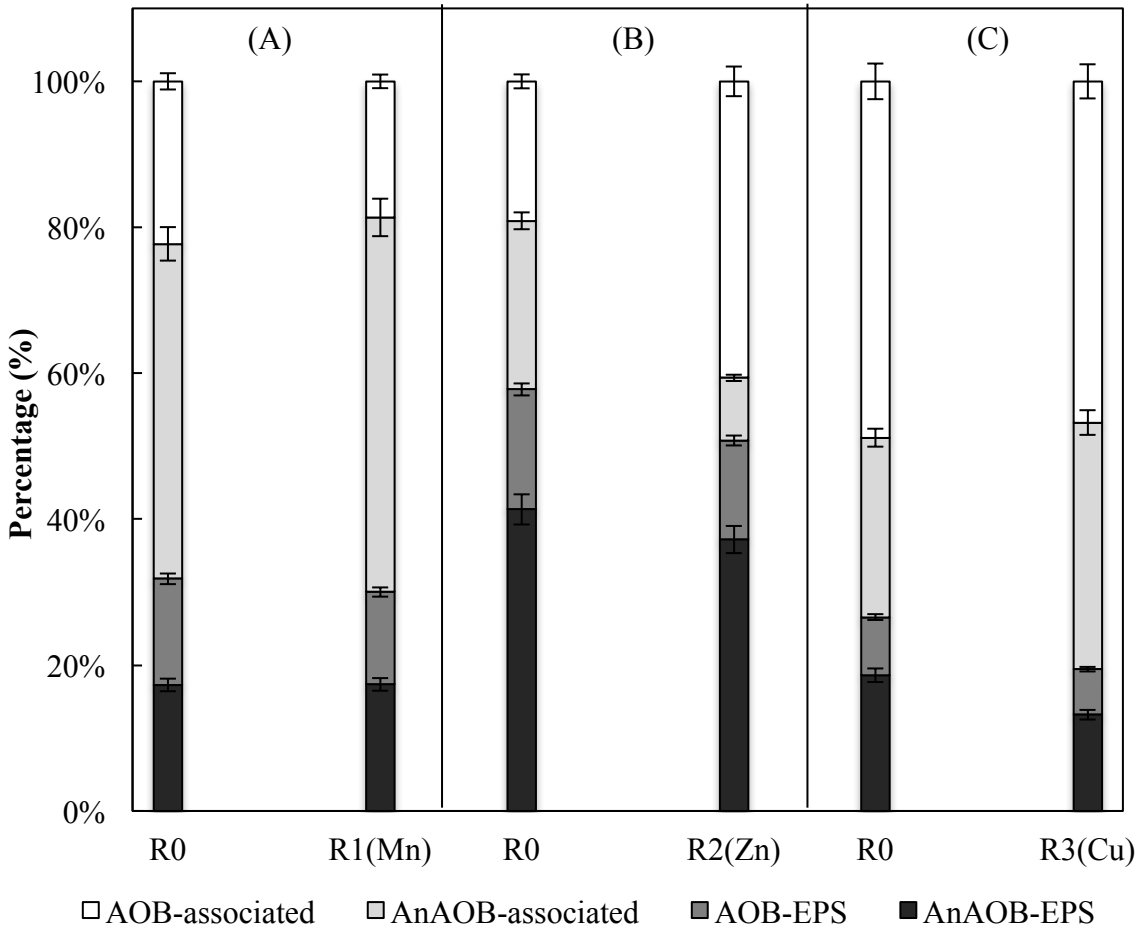

Fig. 6. Distribution of trace elements in bacterial EPS or intracellular components after long-term exposure to trace elements. (A) Mn in control and Mn treatment reactor; (B) Zn in control and Zn treatment reactor; (C) Cu in control and Cu treatment reactor.

Highlights

1. Proper amendment of Mn, Zn and Cu enhances short-term nitritation-anammox performance.
2. Only Mn improves nitrogen removal in long-term nitritation-anammox.
3. Trace elements simultaneously encourage activities of AnAOB and alter microbial community in nitritation-anammox process.
4. Distinct metal impacts explained by distribution in EPS and intracellular components