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Effect of zinc on anammox activity and performance of simultaneous partial nitrification, anammox and denitrification (SNAD) process

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HIGHLIGHTS

- Inhibitory effects of zinc on SAAs were observed.
- The IC_{50} value of zinc was found to be 6.9 mg/L.
- Zinc (<10 mg/L) stimulated the performance of SNAD reactor.
- SNAD reactor showed high treatment efficiencies under high zinc stress conditions.

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ABSTRACT

In the present study, short-term effects of zinc on anammox activities and long-term effect of zinc on the performance of simultaneous partial nitrification, anammox and denitrification (SNAD) process were evaluated. The anammox activity decreased with increasing zinc concentration and exposure time in short-term tests. The IC_{50} value of zinc was found to be 6.9 mg/L. However, the presence of zinc (<10 mg/L) in wastewater stimulated the microbial activities and nitrogen removal performance of SNAD process in sequencing batch biofilm reactor (SBBR). At first, inhibition of SNAD process was observed when influent zinc concentration increased to 20 mg/L. The system recovered immediately, suggesting the acclimatization of microbial communities of SNAD process. The results showed that SBBR was well acclimatized under high zinc concentration (50–100 mg/L) achieving 98% NH_4^+-N, 96% TN and 87% COD removal efficiencies.

1. Introduction

Anaerobic ammonium oxidation (anammox) process, which has multiple advantages over conventional nitrification–denitrification process, has been recognized as the most cost effective biological nitrogen removal processes (Siegrist et al., 2008). Anammox reaction (Eq. (1)) is carried out by a group of planctomycete bacteria, which uses nitrite as the most preferable electron acceptor (Kuenen, 2008; Sabumon, 2007). Anammox is usually combined with partial nitrification, which provides nitrite to anammox bacteria.

$$NH_4^+ + 1.32\,NO_2^- + 0.066\,HCO_3^- + 0.13\,H^+ \rightarrow 0.066\,CH_2O_{0.5}N_{0.15} + 1.02\,N_2 + 0.26\,NO_3^- + 2.03\,H_2O \quad (1)$$

The anammox bacterium has very slow growth rate with a doubling time of 10–11 d and maximum specific growth rate of 0.0027 h^{-1} (Strous et al., 1998; van der Star et al., 2007). As a result, long start-up time is required for anammox or its related processes such as completely autotrophic nitrogen removal over nitrite (CANON) and simultaneous partial nitrification, anammox and denitrification (SNAD). In recent years, much attention has been given on biomass retention using different reactor configurations and biomass carriers, and process inhibition due to substrates (free ammonia, nitrite and free nitrous acid), oxygen, pH and temperature to shorten the start-up time and stable operation of anammox process (Fernandez et al., 2008; Jin et al., 2012; Sri Shalini and Joseph, 2012). The presence of widespread inhibitors such as toxic chemicals, antibiotics and heavy metals in wastewaters can further extend the start-up time of anammox process. However, very few studies have been reported on inhibition of anammox process by heavy metals and antibiotics, which are common in many nitrogen rich wastewaters (Fernandez et al., 2009; Lotti et al., 2012; Zhang et al., 2013).

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Zinc is one of the most common heavy metals found in many nitrogen rich wastewaters (Table 1) including supernatant of anaerobic digested swine waste. The supernatant of anaerobic digested swine waste also contains low organic matter along with high ammonia. Since partial nitrification-anammox or CANON process unable to remove organic carbon and generates nitrate as a byproduct, which further needs to be treated in some cases, this kind of wastewater can be best treated by the SNAD process. In SNAD process, aerobic ammonia oxidizing bacteria (AOB), anammox and denitrifying bacteria co-exist in a single reactor under oxygen limiting conditions and responsible for simultaneous removal of inorganic nitrogen and organic carbon. Three steps can describe the SNAD process. In first step ammonia is partially converted to nitrite by AOB, which consume oxygen and create anoxic conditions. Subsequently in second step, anammox bacteria convert ammonia and nitrite to nitrogen gas and nitrate under anoxic conditions. In third and final step, denitrifying bacteria reduce nitrate produced by anammox bacteria to nitrogen gas by using organic carbon (COD) as reducing agent.

As it is well known that the heavy metals inhibit various microorganisms, it is important to study the effect of zinc on SNAD process. Effect of zinc on nitrification has been studied by few researchers (Hu et al., 2004; Grunditz et al., 1998; Cecen et al., 2010; Madoni et al., 1999). Recently, Feng et al. (2013) studied the effect of heavy metals (zinc, copper, lead and cadmium) on the performance of anoxic/aerobic-membrane bioreactors treating municipal wastewater. The authors concluded that heavy metals more significantly affect nitrification than denitrification. Short-term effect of zinc on the activity of anammox bacteria has been studied recently (Lotti et al., 2012).

In SNAD process, aerobic ammonia oxidizing bacteria (AOB), anammox bacteria and denitrifying bacteria co-exist in a single reactor and the effect of zinc on SNAD process is unknown. Therefore, the main objective of this study was to investigate the long-term effect of zinc on the performance of SNAD process. The short-term effect of zinc on anammox was also evaluated in this study.

2. Methods

2.1. Short-term effect of zinc on anammox activity

Batch experiments were performed to evaluate the short-term effects of zinc on anammox activities. The experiments were carried out as described in Table 2. The non-acclimated microbial sludge used to study the effect of zinc on anammox activity was harvested from the full-scale landfill leachate treatment plant located in Bali, Taiwan. The full-scale treatment plant was operated at 30 °C with average nitrogen loading rate (NLR) of 260 g-N/m^3 d. The concentration of zinc in influent was varied from <1 to <7 mg/L. The acclimated microbial sludge used to study the short-term effect of zinc on anammox activity was harvested from the SBR operated as SNAD process (see Section 2.2 for detail). From the previous study (Daverey et al., 2014), it was observed that the effect of heavy metal is more prominent at low biomass concentration (<2000 mg-MLVSS L^{-1}). Therefore, in this study biomass concentrations were kept below 2000 mg-MLVSS L^{-1} to study the short-term effect of zinc on anammox bacteria. All specific anammox activity (SAA) tests were performed in 67 ml serum bottles according to the previously reported methods (Dapena-Mora et al., 2007; Daverey et al., 2013). The effect of zinc on SAA was measured at 2, 5, 10, 15 and 20 mg/L of zinc concentration for non-acclimated sludge. Zinc (Zn II) added to the serum bottles were in the form of zinc oxide. For acclimated biomass sludge, SAA test was carried out at 20 mg/L of zinc concentration. The SAAs were calculated at different time intervals (3, 6, 12 and 24 h) to evaluate the effect of exposure time. All SAA tests were performed in duplicate. Additional sets of abiotic and negative controls experiments were also carried out for each test.

The short-term effect of zinc on anammox was measured as percentage of activity and calculated as Eq. (2).

$$SAA\ (\%) = \frac{SAA}{SAA_0} \times 100 \tag{2}$$

where SAA_0 is the specific anammox activity in the control assay (in absence of zinc) and SAA is the specific anammox activity of the test sample with zinc.

The IC_{50} is the concentration of the tested compound, which corresponds to 50% activity compared with control assay.

2.2. Long-term effect of zinc on SNAD process

A stirring SBBR (2.5 L) operated at 25 °C and equipped with dissolved oxygen (DO), temperature and pH probes for online monitoring of pH, ORP and DO, respectively was used in this study. Complete mixing inside the reactor was achieved by means of a mechanical stirrer with rotating speed of 150 rpm. Influent was supplied via the top of the reactor using a peristaltic pump. A separate peristaltic pump was used for withdrawing effluent from the reactor. Total 40 carriers (diameter, 2 cm and surface area, 12.6 cm^2/carrier) made from waste activated sludge (WAS), red soil and chemical additive vesicant were arranged around the wall of reactor for biomass attachment and biofilm formation inside the reactor. The WAS, red soil and chemical additive vesicant were dried at between 80 °C and 120 °C. The dried samples of WAS, red soil and chemical additive vesicant were crushed and sieved to obtain powder with particles diameters of about 0.3 mm. It was further ground to fine powder particles of 10 μm or less. The final composition ratio of WAS, red soil and chemical additive vesicant was in the range of 5:6:1 to 5:12:1 (w/w). The mixture was then molded at 1000 °C firing and then cooled to solidify the carriers.

The SBBR was fed with synthetic wastewater supplemented with mineral medium. Ammonium nitrogen (NH_4^+-N) and organic matter (COD) in the synthetic wastewater were supplemented in

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

Wastewater	Zinc concentration (mg/L)	NH_4^+-N concentration (mg/L)	References
Industrial			
Metal plating	30–145	50–600	Stankovic et al. (2009)
Rubber manufacturing	250–300	150–200	Subbiah et al. (2000)
Domestic			
Sewage	0.1–5	20–40	Davis et al. (2001) and Santos et al. (2010)
Other			
Swine	1.5–30	400–800	Nicholson et al. (1999) and Vanotti et al. (2007)
Landfill leachate	0.05–1000	50–2200	Kjeldsen et al. (2002) and Renou et al. (2008)

Table 2
Experimental plan to evaluate the short-term effect of zinc on anammox activity.

Series	Short-term effects of zinc on anammox	Sludge source	Initial MLSS/MLVSS (mg/L)	Zinc concentration (mg/L)	Exposure time (h)
1	Non-acclimated biomass	LLTP[a]	3400 ± 400/1600 ± 200	0, 2, 5, 10, 15, 20	3, 6, 12, 24
2	Acclimated biomass (suspended and biofilm)	SBR[b]	2900 ± 670/1900 ± 520	0, 20	3, 6, 12, 24

[a] Landfill leachate treatment plant, Bali, Taiwan.
[b] SBR (2.5 L) used to study the long-term effect of zinc.

the form of NH_4Cl (ammonium chloride) and glucose, respectively. The composition of the mineral medium used was (g L^{-1} except for trace element solution): $KHCO_3$, 1.25; KH_2PO_4, 0.025; $CaCl_2 \cdot 2H_2O$, 0.3; $MgSO_4$, 0.2; $FeSO_4$, 0.00625; EDTA, 0.00625 and trace element solution 1 mL L^{-1} (Sliekers et al., 2002). The $NaHCO_3$ was added as inorganic carbon source and buffering agent to control pH between 7 and 8. The DO concentration in the reactor was intended to maintain at ~0.1 mg O_2/L. These conditions (temperature, 25 °C; pH, 7–8; DO, ~0.1 mg O_2/L) are suitable for SNAD process. The SBBR was operated in cycles of 24 h. The distribution of each cycle was as follows: 23 h 40 min for reaction (including 6 h for filling), 5 min for settling time and 15 min for decanting. The SBBR was operated for 248 d as SNAD system and its NH_4^+-N, total nitrogen (TN) and COD removal efficiencies (average of last 30 d) were 90%, 88% and 90%, respectively before adding the zinc.

The operational strategy to evaluate the effect of zinc on the performance of SNAD process is shown in Table 3. The whole experiment was divided into six phases (P_1–P_6) in which the influent concentration of zinc was gradually increased from 2 to 100 mg/L. The other reactor operating conditions such as agitator speed, HRT, influent NH_4^+-N concentration, influent COD concentration, NLR and organic loading rate (OLR) were kept constant at 150 rpm, 1.6 d, 600 mg/L, 300 mg/L, 360 g NH_4^+-N/m^3 d and 180 g COD/m^3 d, respectively.

2.3. Measurements and analytical methods

Analytical measurements of mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), NH_4^+-N, NO_2^--N, NO_3^--N and COD were analyzed according to the Standard methods (APHA, 1998). The effluent samples were analyzed twice or thrice per week. The effluent concentration of soluble zinc ions was analyzed by using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). To measure the zinc ions absorbed by the biomass, sludge sample was withdrawn on day 385 from the reactor and pretreated by acid digestion method. The zinc ions in pretreated samples were measured by using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS). A carrier was put in 100 ml of distilled water and oscillated for 10 min to detach the attached sludge on biomass carrier. On-line measurements of pH, ORP and DO were carried out by using pH meter, ORP meter (Suntex PC3200, Taiwan) and DO meter (HACH sc100, Germany), respectively.

Table 3
Zinc concentrations during the six experimental phases.

Phase	Time[a] (d)	Concentration of Zinc in influent (mg/L)	Zinc loading rate (g Zn/m^3 d)
P_1	249–260 (12)	2	1.2
P_2	261–281 (21)	5	3
P_3	282–302 (21)	10	6
P_4	303–330 (28)	20	12
P_5	331–352 (22)	50	30
P_6	353–377 (25)	100	60

[a] Values in parenthesis are the total duration of each phase in days.

3. Results and discussion

3.1. Short-term effect of zinc on anammox activity

In order to apply the anammox and its related processes for the treatment of zinc containing nitrogen-rich industrial wastewaters, it is necessary to know the effect of zinc on anammox activity. Therefore, the SAA tests under zinc stress conditions were carried out as described in Table 2. The initial concentration of zinc was varied between 2 and 20 mg/L and the SAA calculated at different exposure time (3, 6, 12 and 24 h) for non-acclimated biomass sludge. Fig. 1 shows the SAA of non-acclimated sludge as expressed in activity percentage at different concentrations of zinc and incubation time. It is clear from the Fig. 1 that zinc had inhibitory effect on anammox activity at all tested concentrations. Also, the inhibitory effect of zinc on anammox activity increased as concentration of zinc and exposure time increased. The inhibition at 2 mg/L of zinc, the minimum concentration tested in this study at 3 h of exposure was 23% and increased to 37% at 24 h of exposure. In this study, the maximum inhibition was observed at 20 mg/L of zinc with 24 h exposure time. The SAA was only 8% compared to the control at 20 mg/L of zinc after 24 h of exposure time. The IC_{50} of zinc at different exposure time was calculated using the linear regression analysis and the results are described in Table 4. The IC_{50} of zinc was found to be 6.9 mg/L at 24 h of exposure time.

Lotti et al. (2012) also observed similar effect of zinc on anammox activity. However, the inhibition effect on anammox activity was higher as the IC_{50} of zinc was found to be 3.9 mg/L at 24 h exposure time in their study. The inhibition effect of heavy metal depends on biomass concentration and state of microbial growth (Wang et al., 2010). Therefore, the lower IC_{50} of zinc reported by Lotti et al. (2012) was obvious as they used lower biomass concentration to study the inhibition effect of zinc on anammox activity compared to our study. In case of aerobic ammonia oxidizing bacteria (AOB), the IC_{50} of zinc was 6.5 mg/L after 21 h of exposure

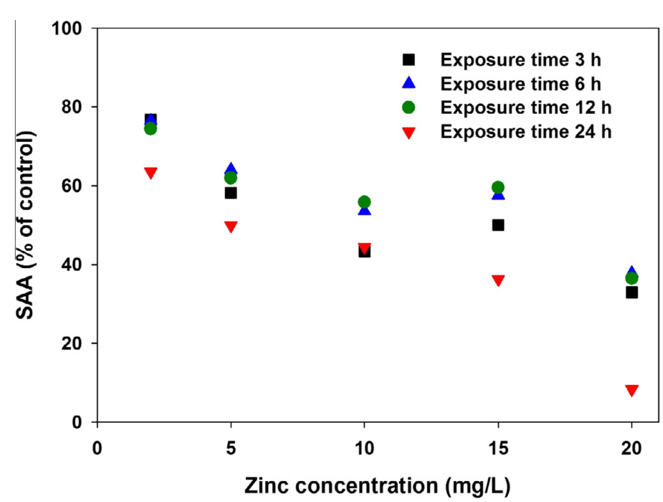

Fig. 1. Short-term effect of zinc on anammox activity.

Table 4
The corresponding IC_{50} of zinc at different exposure time.

Parameter	Value			
Exposure time (h)	3	6	12	24
Zinc IC_{50} (mg/L)	11.5	14.8	14.9	6.9
Regression coefficient (R^2)	0.79	0.85	0.80	0.92

(Cecen et al., 2010), which is almost similar to IC_{50} of zinc on anammox activity observed in present study.

To study the effect of zinc on acclimated biomass, sludge (suspended and sludge attached to the carrier) was harvested from the SBBR operated as SNAD process and SAA tests were performed. The results showed acclimated suspended biomass showed little enhanced SAA at all tested exposure times. At 24 h of exposure time, the SAA of acclimated suspended biomass was 131 ± 15% compared to control. The SAA of acclimated biomass attached to the carrier was slightly inhibited at short exposure time (9% and 6% inhibition at 3 and 6 h of exposure, respectively), however it recovered at 24 h of exposure time. On the other hand, the SAA of non-acclimated biomass was inhibited to 92% after 24 h of exposure time compared to the control.

3.2. Long-term effect of zinc on the performance of SNAD process

The experiment was carried out in a SBBR operated as SNAD process. The SBBR had been operated for 248 d before studying the long-term effect of zinc. The long-term effect of zinc on nitrogen and COD removal efficiencies of SNAD reactor were studied between days 249 and 377 in six phases (P_1–P_6) with different concentrations of zinc in influent wastewater (Table 3). Fig. 2 shows the profiles of DO, pH and ORP in the reactor while Fig. 3 and Fig. 4 show the effluent quality and wastewater treatment efficiency, respectively by the SNAD reactor under zinc stress conditions. The pH in the reactor remained constant at about 7.5 throughout the experiment, while DO (after reaction phase of SBR cycle) and ORP fluctuated between 0 and 2 mg/L, and +100 and −100 mV, respectively during the experiment (Fig. 2).

3.2.1. Stimulation of SNAD process under low concentration of zinc (P_1–P_3)

In P_1, P_2 and P_3, the concentrations of zinc in influent were 2, 5 and 10 mg/L, respectively. The average effluent concentrations of NH_4^+-N and NO_2^--N were 6 and <2 mg/L, respectively in P_1–P_3. Though the effluent concentrations of NO_3^--N were increased from 272 d onwards, the maximum concentration not exceeded 22 mg/L

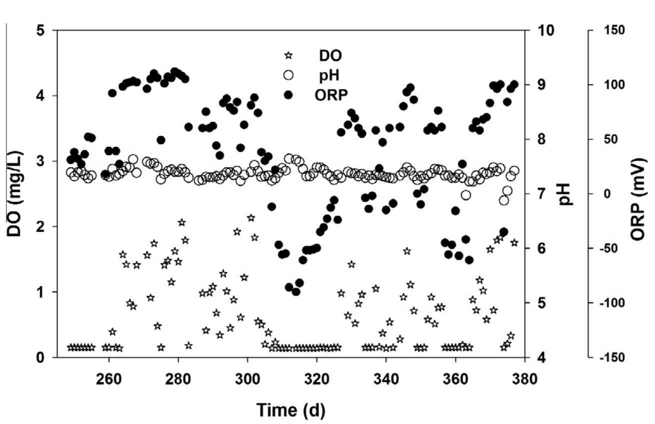

Fig. 2. Temporal variations of pH, dissolved oxygen (DO) and oxidation reduction potential (ORP) in the reactor.

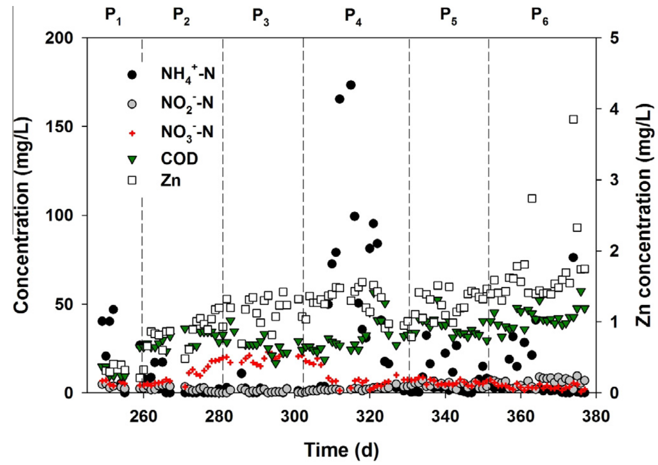

Fig. 3. Temporal variations of inorganic nitrogen compounds, COD and zinc in the reactor effluent.

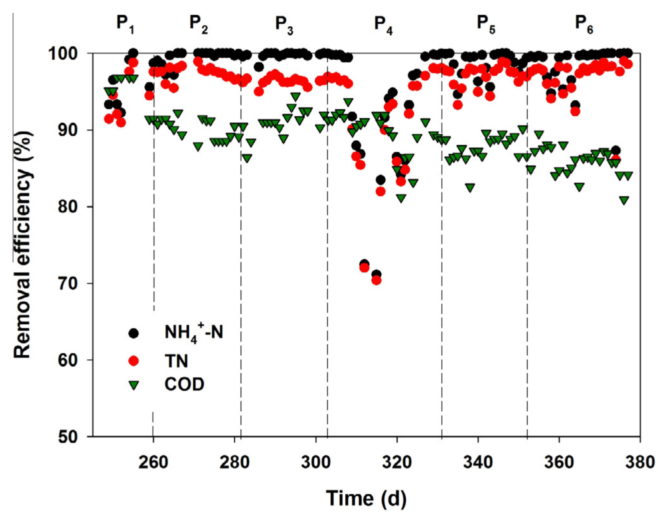

Fig. 4. Nitrogen and COD removal efficiencies of the zinc-stressed SNAD system in different phases.

(Fig. 2). The effluent concentrations of COD were slightly increased from <20 mg/L in P_1 to about 35 mg/L in P_2–P_3. The wastewater treatment efficiency of the SNAD system was remained superior during P_1–P_3 (Fig. 3). The average NH_4^+-N, TN and COD removal efficiencies during P_1–P_3 were 99%, 96% and 91%, respectively, while in absence of zinc these values were 90%, 88% and 90%, respectively. This suggests that the low concentrations of zinc (<10 mg/L) stimulated the ammonia removal efficiency of the SNAD system. This could be explained as zinc binds to the active site of ammonia monooxygenase (a key enzyme of ammonium oxidation which contains 0.5–2.6 mol zinc/mol of enzyme) present in AOB and affects the growth and metabolic activities of AOB (Gilch et al., 2009; Lee et al., 2011). Therefore, ammonium oxidation by AOB was increased in the present study under low concentrations of zinc (<10 mg/L). The influent concentration of zinc was further increased to 20 mg/L in P_4.

3.2.2. Inhibition and recovery of SNAD process under high concentration of zinc (P_4)

In P_4 (303–330 d), the concentration of zinc in influent was increased to 20 mg/L. The ORP was decreased sharply to −90 (Fig. 2) and sudden increase in effluent NH_4^+-N concentration was observed with maximum value of 173 mg NH_4^+-N/L in the middle of P_4 (days 314, 315, Fig. 3). This suggested the inhibition of NH_4^+ oxidation either by AOB, anammox bacteria or both. Therefore, the NH_4^+-N and TN removal efficiencies were sharply decreased to 71% and 70%, respectively on days 309–315 (Fig. 4). While no significant change was observed in the effluent NO_2^--N concentrations, the effluent concentration of NO_3^--N decreased between days 309 and 315. The nitrogen (NH_4^+-N and TN) removal efficiencies of the system were recovered to the previous levels in the second half of the P_4 (315–330 d). In case of COD removal, no negative effect was observed in the first half of the P_4 (303–317 d). Though the COD removal efficiency decreased sharply to 81% on 321 d, the efficiency restored to the previous levels (91%) by the end of P_4.

Recent studies suggested that heavy metals have a significant effect on nitrification than on denitrification (Principi et al., 2006; Feng et al., 2013). While zinc has similar inhibition effects on anammox (reported in this study) and nitrification (Cecen et al., 2010) in short-term tests. In present study nitrite was not accumulated in the reactor (Fig. 3), which suggested that the anammox reaction was not inhibited by addition of zinc. It is believed that sudden increase in zinc concentration temporarily inhibited the partial nitrification (AOB), which affected the anammox reaction. The anammox reaction produced insufficient nitrate for denitrification reaction and therefore decreased the COD removal. However, the reactor performance was recovered to the previous level on day 327, suggesting that microbial communities of the SNAD system were well acclimated to the zinc.

3.2.3. Stable performance of SNAD reactor under very high concentration of zinc (P_5 and P_6)

In P_5 and P_6 (331–377 d), the reactor performance under high zinc concentrations (50 and 100 mg/L) was investigated. Fig. 3 shows that the effluent quality of wastewater remained consistent in P_5 and P_6. Fig. 4 shows that the nitrogen and COD removal efficiencies of the SNAD system remained stable under high concentrations of zinc. The average NH_4^+-N, TN and COD removal efficiencies were 98%, 96% and 87%, respectively between days 331 and 377 (P_5 and P_6). Overall, the experimental results suggest that SAND process can acclimatize and successfully treat high zinc containing nitrogen-rich wastewaters such as swine waste.

3.2.4. Adsorption of zinc on biomass

The soluble concentrations of zinc in the effluent of SBR were <2 mg/L throughout the experiment (Fig. 3). The suspended biomass and biomass attached to the carrier were harvested from the reactor at the end of experiment (day 377) to analyze the amount of zinc adsorbed on the biomass sludge and the results are presented in Table 5. It was observed that the zinc adsorption capacity of attached biomass was more than the suspended biomass. However, the zinc adsorption capacities by suspended and attached biomass in this study were within the range of the highest metal adsorption capacities of the microorganisms (5–640 g metal/g dry matter) as suggested by Ahluwalia and Goyal (2007). According to the mass balance of zinc, total zinc fed to the reactor (6752 mg) is the sum of soluble zinc out in effluent (227 mg), zinc out as SS (350 mg), zinc adsorbed on biomass attached to the carrier (925 mg), zinc adsorbed on biomass (1200 mg) and zinc accumulated in the reactor (4050 mg). Consequently, it exhibited that the major portion of zinc remained inside the reactor.

Table 5
Adsorption of zinc on the attached and suspended biomass of the SNAD reactor.

Sludge	Amount of biomass in the reactor (g)	Zinc adsorbed (mg/g of dry matter)	Total amount of zinc adsorbed (mg)
Attached (biofilm)	10	92.5	925
Suspended	20	59.7	1194

4. Conclusions

Short-term tests suggest that zinc has inhibitory effect on anammox activity under tested conditions. The low concentrations of zinc (up to 10 mg/L) had positive effects on ammonium oxidation and thereby the improved nitrogen removal efficiency of the SNAD reactor was observed. Temporary inhibition of SNAD process was observed at 20 mg/L of zinc concentration. The SNAD system had shown resistance to the high concentration of zinc (up to 100 mg/L) after acclimatization of biomass.

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