Insight on the microbial activity and microbiome in partial nitrification systems: CuO nanoparticles impact under different pH levels

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ABSTRACT

In this study, the effect of CuO nanoparticles (NPs) on partial nitrification (PN) process was investigated at various pH values. The short- and long-term experiments were carried out in six identical reactors, with or without CuO NPs, at pH values of 6.5, 8.0 and 10.0. The ammonia oxidation, reaction rates, copper content, and the microbial community were investigated. The results of this work suggested that CuO NPs inhibited the ammonia oxidation by aerobic ammonia-oxidizing bacteria (AOB), under both the acid and alkali conditions. AOB could resist the acid condition but was significantly suppressed when CuO NPs was fed, and the low pH did not aggravate the inhibition level. Almost all the bacteria lost bioactivity under the pH as 10.0, while anaerobic ammonia-oxidizing bacteria survived. The acid condition increased the *Nitrosomonas* relative abundance while the alkali condition lowered it. More than 60% of the supplied CuO NPs was discharged via effluent.

Keywords: AOB, CuO NPs, Nitrogen removal, Partial nitrification, pH

1. Introduction

Partial nitrification (PN) is the necessary upstream process for the energy efficient anaerobic ammonium oxidation (Anammox) nitrogen removal processes [1]. In the PN process, ammonia nitrogen is oxidized to nitrite by the aerobic ammonia-oxidizing bacteria (AOB), while the activity of the nitrite-oxidizing bacteria (NOB) is effectively suppressed. This process leads to the accumulation of nitrite and provides the produced nitrite necessary for the subsequent Anammox process [2]. Therefore, the stable operation of PN plays an important role in Anammox-based autotrophic nitrogen removal systems. However, it should be noted that PN processes has been shown to be susceptible to many operational factors, such as temperature [3], pH [4], free ammonia (FA) concentration, high free nitrous acid (FNA) concentration [5], dissolved oxygen (DO) concentration [2]; which in-turn limited the performance of the subsequent Anammox system and/or the hybrid PN-Anammox system [6-8].

Nanoparticles (NPs) are an emerging group of contaminant that has increased in their accumulation in waters in recent years due to their wide use in numerous industries; including the medical and pharmaceutical [9], engineering [10], electronics, optics, chemical sensors, and biosensors [11] industries. NPs that have inevitably been released to the environment [12] have been identified and measured in numerous wastewater treatment systems [13]. Their presence has demonstrated a significant impact on the treatment efficiency of wastewater treatment systems [13, 14]. CuO NPs is one of the most widely used NPs, which is produced in various industrial processes, such as the preparation of anti-infective materials [15] and catalysts [16]. Several studies have investigated the toxic effects of CuO NPs on water and wastewater treatment systems; such as natural water environments [17], activated sludge wastewater treatment systems [18], nitrogen and phosphorous wastewater removal systems [19], anammox wastewater treatment systems [7, 20], as well as the PN process used in wastewater treatment [21]. However, a gap of knowledge still exists in our understanding

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of CuO NPs effects on these systems at differing pH values. Upon entering the environment or wastewater treatment system, NPs may aggregate, settle or release Cu (II) [10, 14, 22, 23], with the fate of the NPs being dependent on the pH of the bulk liquid. Acidic conditions may increase the release of Cu (II) by CuO NPs, while copper hydroxide may be formed under alkaline conditions [22, 24]. Since the state of CuO NPs is significantly affected by pH and the state of the NPs directly influences their subsequent effects on biological wastewater treatment system, it is necessary to enhance our understanding of CuO NPs on the PN process at various pH values.

In this study, the short- and long-term impacts of CuO NPs at various pH values on the fate of the NPs, the nitrogen removal performance and the microbial community of the PN system were investigated. The results of this work provide new information with respect to the fate of CuO NPs that enter sewage treatment system and the subsequent effects on PN processes.

2. Material and Methods

2.1. Experimental Setup

A parent sequencing batch reactor (SBR) identified as R0 was used to supply the inoculum sludge for the design phase of this study. R0 was operated as a high-rate PN process for longer than one year. For the present experiment, the sludge in R0 was equally divided into six reactors, respectively named R1, R2, R3, R4, R5, and R6, with effective volumes of 1 L each, as shown in Fig. 1. The initial mixed liquor suspended solids (MLSS) of each reactor

was 2.5 g/L. The operational conditions of R1 were identical to the parent reactor R0, thus R1 was operated as a control reactor. The operational difference between reactors R2, R3, R4, R5, and R6 was solely the rate of CuO NPs addition and the operational pH, as shown in Table 1.

CuO NPs in 1 g/L stock solution was prepared by adding 1 g NPs (40 nm) into 1 L Mill-Q water. The stock solution was exposed to ultrasonic dispersion for 30 min. Following ultrasonic dispersion, the solution (1 mL) was directly added to reactors R2, R4, and R6. The influent pH for R1 and R2 was set as 8.0, R3 and R4 as 6.5, and R5 and R6 as 10.0. Each pair of reactors were compared to analyze the CuO NPs effect at pH values of 6.5, 8.0 and 10.0, to consider the accident condition during the wastewater production process. In PN process, ammonia oxidation could not be carried out when the pH is lower than 6.5 since the alkalinity was insufficient [25]. The air flow rate of the six reactors was constant and identical, being set at 0.1 L/min. The DO concentration in each reactor was different due to varying microbial activities in the reactors and hence varying oxygen consumption rates.

The synthetic wastewater contained (in g/L): $(\text{NH}_4)_2\text{SO}_4$ 0.942, NaHCO_3 2.685, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.150, CaCl_2 0.068, KH_2PO_4 0.068 and 1 mL/L trace element solution. Feed ammonia was added to achieve a concentration of 200 mg/L at the start of each sequencing cycle. The ammonia concentration in each reactor was different due to residual ammonia remaining from the last cycle. NaOH and HCl in 1 M were used to adjust the pH in the feed of each reactor. Other operational conditions were summarized as Table 1.

Short and long-term experiments were respectively carried out in this study. For the short-term experiment, the reaction rates

Fig. 1. Experimental device. (a) Power switch (b) Speed controller (c) Airflow meter (d) Stirrer (e) Aeration ring (f) Aeration pump.

Table 1. Operational Conditions and the Performance of Each Reactor (average data during the whole experiment)

Reactor	Inf. $\text{NH}_4^+\text{-N}$ (mg/L)	pH	Aeration rate (L/min)	DO (mg/L)	T (°C)	ARE (%)	TRE (%)
R1	167.6	7.94	0.1	0.21	17.0	80.4	5.0
R2	176.6	7.91	0.1	0.20	17.2	45.8	18.1
R3	172.1	6.57	0.1	0.24	17.7	60.1	12.4
R4	180.2	6.53	0.1	0.20	17.1	45.3	18.0
R5	167.4	9.93	0.1	7.64	18.3	41.8	40.9
R6	168.3	9.93	0.1	7.59	18.5	42.6	41.6

under different conditions were detected in each reactor. The operational conditions were the same as the long-term experiment, and the reaction time was 4 h. The experiment was triplicately carried out, and the average results were used to calculate the reaction rates. After each experiment, the sludge was washed using synthetic wastewater. The ammonia removal rate (ARR), nitrite production rate (NPR), and TN removal rate (TRR) during the short-term experiment were calculated as follows:

$$ARR = \frac{[NH_4^+-N]_{inf.} - [NH_4^+-N]_{eff.}}{MLSS \times t} \quad (1)$$

$$NPR = \frac{[NO_2^- - N]_{eff.} - [NO_2^- - N]_{inf.}}{MLSS \times t} \quad (2)$$

$$TRR = \frac{[TN]_{inf.} - [TN]_{eff.}}{MLSS \times t} \quad (3)$$

The long-term experiment was conducted for 60 cycles. Each cycle was comprised of the following stages: feed fill for 5 min, reaction 8 h, settling 0.5 h, standby 2 h. The volumetric exchange ratio for each cycle was 80%. Ammonia removal efficiency (ARE) and total nitrogen (TN) removal efficiency (TRE) during the long-term experiment were calculated as Eq. (4) and (5).

$$ARE = \frac{[NH_4^+-N]_{inf.} - [NH_4^+-N]_{eff.}}{[NH_4^+-N]_{inf.}} \times 100\% \quad (4)$$

$$TRE = \frac{[TN]_{inf.} - [TN]_{eff.}}{[TN]_{inf.}} \times 100\% \quad (5)$$

2.2. Analytical Method

Concentrations of NH_4^+-N and $NO_2^- - N$ were measured daily using colorimetric methods and $NO_3^- - N$ was analyzed using the ultraviolet spectrophotometric method. The temperature, DO and pH were detected using portable instruments with specific probes (WTW, Germany).

2.3. Detection of CuO NPs Distribution

The CuO distribution was measured in the following phases, (a) copper in the effluent, (b) copper in the soluble microbial products (SMP), (c) copper content in extracellular polymeric substances (EPS) and (d) copper in sludge. The mixed sludge liquor (10 mL) at the end of the experiment was obtained and settled for 0.5 h, then 8 mL effluent was obtained for measurement of copper in the effluent. The remained sludge was centrifuged at 8000 rpm for 5 min, then 1 mL liquor was obtained for detecting the copper in the SMP. Then 9 mL PBS was added to the sludge and heated at 80°C for 0.5 h, to extract the EPS, the obtained EPS was used for detecting the copper content in the EPS. The remained sludge was digested using strong acid and used to detect the copper content in sludge. The copper content in the four components was determined using atomic absorption spectroscopy (AAS-5000, Germany) after multiple dilutions. The calculated copper contents

in each component were added together to determine the copper content per sludge mixed liquor, by using the quantity in each component divided by the volume of 10 mL.

2.4. Batch Experiments to Determine TN Removal in R5 and R6

To verify the TN removal mechanism in R5 and R6 at high pH, batch experiments were performed at the end of the long-term experiments of R5 and R6. A control batch reactor was operated in addition to R5 and R6. The operation of the control reactor was identical to R5 and R6, however, R0 was not inoculated with activated sludge. The three reactors were mixed with aeration for 8 h, after being supplied with the same feed like that for the long-term experiments. The experiment was triplicated and the average influent and effluent data were used to determine the TN removal.

2.5. Statistical Analysis and Graphical Works

Statistical analyses and graphs were created using Excel and Origin 8.6. The short-term results are presented as means $\pm$ SD (standard deviation of means). Variances of different treatments were analyzed using one-way analysis of variance (ANOVA).

2.6. Microbial Community Detection by High-throughput Sequencing

Sludge sample was obtained from the reactor at the end of the long-term experiments on cycle 60. The DNA of each sludge sample was extracted and qualified, before sequenced by Sangon Company in Miseq sequencing platform (Illumina, Inc., San Diego, CA, USA). The obtained sequences were compared in the SILVA database (citations). OTU was defined as the sequences with the similarity higher than 97%.

3. Results and Discussion

3.1. Short-term Effect of CuO NPs and pH on the Microbial Bioactivity of PN System

The ARR, NPR, and TRR under different conditions are shown in Fig. 2. Compared R1 with R2, exposure in NPs led to a decrease of both ARR and NPR, and a slight increase of TRR. Similar results with respect to a decrease in ARR and NPR, as well as the increase in TRR, were obtained in R3 and R4. However, in R5 and R6 with the feed pH at 10.0, CuO NPs demonstrated a different effect on the nitrogen performance of the reactors. In particular, higher ARR was observed in R6 than R5, while the NPR in both reactors was similar. This result indicates that the high pH conditions of the reactors modified the CuO NPs effect on nitrogen removal performance.

The nitrogen removal performance of R1, R3, and R5 were compared to evaluate the pH effect, while R2, R4, and R6 were compared to analyze the combined effects of pH and CuO NPs. Compared R1, R3, and R5, the ARR in each reactor was 3.05, 1.90 and 1.89 mg N/h/g SS, while the NPR was 3.02, 1.60 and 0.16 mg N/h/g SS. ARR in R1 was the highest, this was attributed to the pH is most optimal for the AOB population, as proved in several previous

Fig. 2. Reaction rates under different condition in the short-term experiment.

studies [21, 26, 27]. It was notable that although the ammonia removal in R3 and R5 were similar, the NPR was very low in R5. The TRR result suggested that the nitrite was consumed or no nitrite was produced. For TN removal, the TRR in R1, R3, and R5 was 0.04, 0.35 and 1.73 mg N/h/g SS, respectively, suggesting the significant increase in TN removal at high pH as 10 in R5. It was interesting that the TRR was approximately to the ARR, while NPR was very low, so the ammonia removal was likely attrib-

uted to ammonia stripping as the pKa of ammonia is 9.25.

The nitrogen removal performance in R2, R4 and R6 were similar in trend to the performance of the R1, R3, and R5 reactors; with the ARR of R2, R4, and R6 being 2.45, 1.60 and 2.1, the NRP being 2.34, 0.70 and 0.16, and the TRR being 0.17, 0.95 and 1.95 mg N/h/g SS, respectively. The ARR in R2 was the highest, while the TRR in R6 was the highest. Moreover, the NPs appear to promote TN removal, which could be attributed to the NPs enhancing the activity of the anammox (AnAOB) population that consume nitrite. Even though the TN removal in each reactor with NPs was higher than that without NPs, the effect of NPs was lighter than that of the pH. The low pH of 6.5 also improved the TN removal, which was likely due to Cu (II) release under acidic conditions that enhance the AnAOB activity. These results suggest that both acid and alkali pH decrease the ammonia oxidation, but lead to higher TN removal. NPs decreased the ammonia oxidation at pH values of 6.5 and 8.0, while increasing ammonia oxidation at a pH of 10, likely due to the free ammonia stripping at alkali pH values. The mechanism related to CuO NPs and pH effects still required further study, hence the long-term experiments of this study was performed.

3.2. Long-term effect of CuO NPs at Varying pH on Nitrogen Removal Performance

The nitrogen components and the ARE results during the 60 cycles of the long-term experiments are shown in Fig. 3. In R1, the ammonia removal performance did not change significantly during the whole

Fig. 3. Reactor performance under different condition.

experiment. The initial ammonia concentration in each cycle was on average approximately 167.6 mg/L, while the ammonia, nitrite, and nitrate concentrations in the effluent were 32.9, 151.8 and 3.3 mg/L, respectively. Most ammonia was oxidized to nitrite, only a small amount of nitrate was produced, indicating sufficient activity of the AOB populations. The average ARE in R1 was approximately 80.4%, while the TRE was limited to approximately 5.0%. In R2, the influent ammonia was approximately 176.6 mg/L, it was higher than that in R1 because more ammonia remained in the reactor at the end of each cycle.

CuO NPs (1 mg/L) was added to the reactor R2 during the feeding stage of each cycle with the reactor pH conditions being 8.0. As a result, the effluent ammonia, nitrite, and nitrate in R2 varied from approximately 95.9, 62.8 and 3.0 mg/L. The average ARE and TRE was approximately 45.7% and 18.1%, respectively. Comparing R1 with R2, the obvious difference was the lower ARE and higher TRE in R2. On one hand, the CuO NPs addition likely caused lower AOB activity, while it induced the AnAOB activity, which consumes the ammonia and nitrite and a small portion of TN as result. This result was inconsistent with our previous study which demonstrated that 1-10 mg/L CuO NPs enhanced AOB activity [21], in which the MLSS was 3.7 g/L and the exposure time in each cycle was 3 h, while those in the present study was 2.0 g/L and 8 h, respectively. Thus, longer exposure times relative to sludge loading conditions may induce lower resistance to CuO NPs.

In R3, the reactor demonstrated different ammonia removal performance in the early and late stages of the experiment. In the early stage, the effluent ammonia was quite elevated, which decreased the ARE to approximately 48.2%. However, with the operation, the PN biomass acclimated to the acid condition and removed more ammonia, demonstrating that the AOB activity recovered as result. This finding shows that the AOB population survive the more acid conditions, even though there were initially suppressed. Moreover, the TN removal in the early 32 cycles was approximately 16.8%. It was notable that the effluent pH increased to about 7.5, which may be indicative of anammox activity. The DO concentration suddenly increased to approximately 0.3 from 0.18 mg/L from cycle 33 onwards. This increase is likely attributed to a microbial death phase, which then lowered the oxygen consumption under the same oxygen supply of 0.1 L/min air. As a result, the activity of the AOB population was improved while that of the AnAOB population was limited. At the later stage of the experiment in R3, the ammonia, nitrite, and nitrate in the effluent was 47.1, 134.7 and 3.2 mg/L, respectively, while the ARE and TRE changed to 72.6% and 7.3%.

In R4 with CuO NPs being fed to the reactor, the nitrogen removal performance was relatively stable. The feed ammonia concentration was approximately 177.9 mg/L, and the ammonia, nitrite, and nitrate in the effluent was approximately 97.3, 60.9 and 2.9 mg/L. The ARE and TRE were approximately 45.3% and 18.0%, respectively. Comparing R3 and R4, it is observed that the ARE in R4 was lower while the TRE was higher. CuO NPs again demonstrated potential inhibition of the AOB population under acid conditions, with the results being similar to that of R2. The results in R3 and R4 suggest that the AOB remain viable during acid conditions, but could not remain viable when exposed in CuO NPs under the acid condition. Furthermore, it is likely that cell death with

CuO NPs addition induced denitrification or the release of Cu (II) that in turn induced anammox in R3.

In R5 and R6, the two reactors demonstrated similar nitrogen removal performance. The influent ammonia was respectively 168.1 and 168.9 mg/L. During the experiment, the nitrogen components in the effluent in the two reactors were relatively stable. The ammonia, nitrite, and nitrate in the effluent of R5 were approximately 97.7, 0.9 and 3.2 mg/L, while those in R6 were 96.9, 0.6 and 3.4 mg/L. The ARE and TRE in R5 was 41.8% and 40.9%, and those in R6 were approximately 42.6% and 41.6%. The results suggest that CuO NPs under alkali conditions had a slight additional influence on the PN system, with the two reactors showing similar ammonia oxidation and TN removal. However, it was notable that the TRE was much higher in R5 and R6 when compared with the results in the other four reactors. No nitrite accumulated in the reactors during the experiment. The TRE was similar to ARE, indicating that all the ammonia was removed instead of accumulating as nitrite in the reactor. Additionally, after the long-term experiment, the sludge in R5 and R6 turned to be earthy yellow, indicating the poor activity of the microorganisms. As shown in Table 1, the DO in R5 and R6 were much higher than other reactors when the aeration rate for each reactor was identical. It was attributed to that AOB lost its activity and could not oxidize ammonia using oxygen. As a result, no oxygen was consumed but remained in the reactor and manifested as high DO. The high DO reflected the poor oxygen consumption and further proved the poor bioactivity of AOB.

Considering the FA (NH₃) smell released from R5 and R6 during the experiment, it was speculated that the ammonia was removed via FA production and stripping. To verify this speculation, batch experiments performed in a control reactor, without activated sludge, were conducted, and the results are shown in Fig. S1. It could be seen that the ARE was 38.8%, 40.7%, and 42.7%, respectively in the control, R5, and R6, while the TRE was 38.1%, 40.0%, and 42.0%. That is, the ammonia removal in R5 and R6 was not statistically significantly different from the control and hence the ammonia removal in R5 and R6 is not attributed to the ammonia oxidation. The fact is that the high pH limited the AOB bioactivity but induced the AnAOB. Since little nitrite could be supplied to AnAOB, the TN removal was limited. As a result, only a small proportion of ammonia was removed by the AnAOB population. The elevated percent abundances of the AnAOB population in R5 and R6 (shown below) support this inference. Additionally, the TRE in R6 was higher than that in R5, with similar results being observed in R2 and R4. As such, the CuO NPs was shown to support the growth of the AnAOB population relative to the microbiome.

On the other hand, comparing the results with R1, R3, and R5, it is evident that elevated pH values exhibit the most significant inhibition on the PN system. Comparing the results with R2, R4, and R6, the NPs fed to the system inhibited both the nitrogen removal in both R2 and R4 due to the high NPs load for per unit sludge, while the elevated pH conditions resulted in almost all the microbial activity being lost.

3.3. CuO Distribution in the System

The copper concentrations of each component of the sludge of

the PN systems were measured (Fig. 4). In seed sludge of R0, the copper content in the effluent, SMP, EPS, and sludge was respectively 0.41, 0.13, 1.88 and 2.54 mg/L. While the copper concentration in R1 was 0.44, 0.17, 2.40 and 3.67 mg/L, in R2 it was 1.01, 0.17, 3.75 and 11.23 mg/L, in R3 it was 0.45, 0.17, 2.42 and 3.71 mg/L, in R4 it was 1.10, 0.18, 2.49 and 8.35 mg/L, in R5 it was 0.46, 0.17, 2.48 and 3.46 mg/L, in R6 it was 0.96, 0.18, 2.58 and 13.99 mg/L. It could be concluded that the CuO NPs addition led to the copper concentration increases in both EPS and sludge, which was especially obvious in the sludge. That is, the CuO NPs addition led to the greatest copper accumulation in the sludge. The copper content in the sludge of R6 was the highest, and this is attributed to the fact that some copper accumulated in the reactor as a precipitate. Correspondingly, the copper concentration in R4 was the lowest, while the copper content in the effluent of R4 was the highest. The acidic conditions of R4 resulted in more copper ions exiting the system via the effluent. For the copper concentrations in the EPS, both the acid and alkali conditions led to a decrease in the EPS concentration, which also suggests that the EPS could help resist CuO NPs shock in R4 and R6.

The mass balance of copper was analyzed in the six reactors. Examining R1 as an example demonstrated that after CuO NPs entered into the reactor, the copper partitioned to the following component of the system:

- Effluent copper, calculated as a

$$= (C - C_0) \times 0.8 \text{ L} \times 60 = (0.44 - 0.41) \times 0.8 \times 60 = 1.61 \text{ mg};$$
- SMP copper, calculated as b

$$= (C - C_0) \times 1 \text{ L} = (0.17 - 0.13) = 0.04 \text{ mg}$$
- EPS copper, calculated as c

$$= (C - C_0) \times 1 \text{ L} = (2.40 - 1.88) = 0.52 \text{ mg}$$
- Sludge copper, calculated as d

$$= (C - C_0) \times 1 \text{ L} = (3.67 - 2.54) = 1.12 \text{ mg}$$

The ratio of each component was calculated as the individual component relative to the sum of the four components (Table 2). It could be seen that in R1, 48.88% of the supplied copper exited via the effluent, while another about 50% was adsorbed by the SMP, EPS or utilized by the microbial cell in sludge. However in R2, with the CuO NPs addition, the ratio in the effluent increased to 73.12%, that is only about 27% was removed by the system. This result was consistent with previous studies that demonstrated that approximately 50% NPs were removed by the wastewater treatment system [14, 28]. Additionally, the copper in the effluent of R3 and R5 were both higher than in R1, indicating less copper was adsorbed by the sludge. This is consistent with the low activity of the nitrogen removal microbial population in R1.

In R2 with a pH of 8.0, 73.1% CuO NPs exited the system, while 0.1% partitioned to the SMP, 4.76% in EPS and 22.0% in the sludge. The results of R4, with a pH of 6.5, were respectively 83.79, 0.13, 1.55 and 14.54, while in R6, with a pH of 8.0, the results were 68.64, 0.13, 1.82 and 29.42. In all three reactors, most of the CuO NPs exited the system via the effluent, with the second largest component showing copper being the sludge. Moreover, in R4, the concentration in the effluent was the highest. The elevated concentration in the effluent is attributed to the fact

that most of the CuO NPs exited from the reactor as Cu (II) and hence was released under acidic conditions. That is, the acidic conditions were shown to not be optimal for CuO NPs removal. Although the CuO NPs concentration in the feed has been shown to result in more EPS production [29], the EPS adsorbed a very small concentration of NPs while a greater quantity was partitioned into the microbial cell of the sludge. As a result, the NPs likely suppressed the microbial activity of the systems. In conclusion, the acid condition led to more CuO NPs dissolution, while the alkali condition led to more NPs aggregated and accumulated in the sludge.

Fig. 4. Metal content in the four different parts of each reactor.

Table 2. The Copper Content Shared Ratio in Each Part of the System

Reactor	The ratio of a* part	The ratio of b* part	The ratio of c* part	The ratio of d* part
R1	48.88	1.28	15.75	34.09
R2	73.12	0.10	4.76	22.02
R3	56.48	1.01	13.48	29.03
R4	83.79	0.13	1.55	14.54
R5	62.62	1.07	14.36	21.94
R6	68.64	0.13	1.82	29.42

* a. copper in effluent, b. copper in SMP, c. copper in EPS, d. copper in sludge

3.4. Microbial Community Results Under Different Condition

Sludge samples were collected from the six reactors and analyzed for their microbial community. As shown in Table 3, 47651, 53119, 55908, 50006, 58550 and 55929 sequences were detected from each reactor, which was defined as 722, 869, 903, 870, 1005 and 960 OTUs, respectively. Both the acidic and alkali conditions led to the OTUs increase, while the CuO NPs addition led to decrease. The Shannon index showed the same result, which was 3.46, 3.48, 3.66, 4.22, 4.43 and 4.42, respectively. It increased in both R3 and R5 when compared with R1, that is the pH variation led to the biodiversity increase of the microbial community. It was attributed to some acid-resisted bacteria or alkali-resisted bacteria survived in the reactors. Similarly, the CuO NPs also resulted in the

Table 3. Detailed sequencing information and the relative abundance of several functional bacteria in each reactor

Sample	Detected sequences	OTU numbers	Shannon index	Nitrosomonas (%)	Candidatus_Kuenenia (%)	Denitratisoma (%)
R1	47651	722	3.46	11.83	0.25	1.25
R2	53119	869	3.48	18.93	0.46	1.86
R3	55908	903	3.66	43.79	0.41	0.91
R4	50006	870	4.22	18.63	0.64	3.08
R5	58550	1005	4.43	8.13	1.9	2.42
R6	55929	960	4.42	7.55	3.68	3.05

Fig. 5. Taxonomic result in genus level of each reactor.

higher biodiversity, which was likely due to the Cu (II) or NPs enabling new microbial populations to compete [30].

The similarity between every two reactors was summarized in Table S1. It could be seen from the Beta diversity result that the similarity between R1 and R2 was 0.90, while those in R1 and R3, and R1 and R5 was only 0.67 and 0.64, respectively. That between R2 and R4, R2 and R6 were only 0.80 and 0.66. The relative low similarity suggested that the pH variation led to the significant different shift of the microbial community components, while the acid condition performed slighter effect with CuO NPs addition. In addition, the similarity between R3 and R4 was 0.76, while that between R5 and R6 was 0.88, which was higher than the reactors with different pH. This result suggested that the CuO NPs performed lower influence than the pH condition on the microbial diversity, and it performed the lowest effect in alkali condition.

The first fifty bacteria in the genus level are shown in Fig. 5, and the relative abundance of the nitrogen removal-related bacteria are summarized in Table 3. Combined with Fig. 5 and Table 3, it could be concluded that *Nitrosomonas* was the predominated AOB genus in the six reactors. The relative abundance was 11.83%, 18.93%, 43.79%, 18.63%, 8.13% and 7.55%, respectively in R1, R2, R3, R4, R5 and R6. Compared with R1, the percentage of *Nitrosomonas* in R2 increased, but the ARE was lower. This may be attributed to the CuO NPs leading to the death bacterial populations, resulting in the relative abundance of AOB increasing as result. In conclusion, the CuO NPs appear to have inhibited specific populations of the microbial community, while the AOB population demonstrated a higher resistance. The relative abun-

dance significantly increased in R3, which was consistent with the reactor performance, it could be attributed to the higher DO that was profitable for AOB. The AOB populations were shown to be resistant to these conditions, with its relative percent abundance increasing as result. The lower abundance in R4 than R3 further indicated the suppression of the PN process by CuO NPs, similar to what was demonstrated in R2. In R5 and R6, AOB was severely suppressed, almost all the microbial activity was lost. Correspondingly, the AOB relative number decreased in both R5 and R6. It was consistent with the reactor performance that almost all of the ammonia was removed via FA production but not by AOB. A small proportion of TN was removed by AAOB or denitrifiers in R5 and R6, indicating that the two kinds of bacteria could resist the high pH, but there was not enough substrate for their utilization. It was notable that *Candidatus_Kuenenia* and *Denitratisoma* were also detected in the six reactors, which afforded the TN removal work. In the three reactors with CuO NPs addition, both the relative number of AAOB and denitrifying bacteria increased, also indicating the positive effect on these two bacteria.

4. Conclusions

The ammonia oxidation of the PN system was significantly suppressed by CuO NPs, in both acid and alkali condition. AOB could resist acid condition (pH=6.5) without CuO NPs, and the low pH did not increase the NPs toxicity. Under pH as 10.0, almost all the microorganisms lost bioactivity, most of the nitrogen removal

was attributed to the production of FA, and the relative abundance of *Nitrosomonas*-AOB decreased to 7.55%. More than 60% of the NPs was discharged via effluent. High sludge concentration is essential when treated the wastewater contains CuO NPs.

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Author Contributions

X.Z. (Associate professor) designed the study and wrote the manuscript. N.Z. and Y.Z. (Master students) conducted all the experiments. R.D. (Associate professor) revised the manuscript and improved the English writing; Y.S. (Lecturer) and Y.M. (Associate professor) analyzed data and draw Figures. H.Z. (Professor) contributed to refining the ideas.

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