Long-term effects of silver nanoparticles on performance of phosphorus removal in a laboratory-scale vertical flow constructed wetland

Juan Huang1,⁎, Jun Xiao1, Yang Guo2, Wenzu Guan1, Chong Cao1, Chunni Yan1, Mingyu Wang1

1. School of Civil Engineering, Southeast University, Nanjing 211189, China
2. Security Support Center for Urban Water Supply of Jiangsu Province, Nanjing 210036, China

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ABSTRACT

Silver nanoparticles (AgNPs) have been widely used in many fields, which raised concerns about potential threats to biological sewage treatment systems. In this study, the phosphorus removal performance, enzymatic activity and microbial population dynamics in constructed wetlands (CWs) were evaluated under a long-term exposure to AgNPs (0, 50, and 200 μg/L) for 450 days. Results have shown that AgNPs inhibited the phosphorus removal efficiency in a short-term exposure, whereas caused no obviously negative effects from a long-term perspective. Moreover, in the coexisting CW system of AgNPs and phosphorus, competition exhibited in the initial exposure phase, however, cooperation between them was observed in later phase. Enzymatic activity of acid-phosphatase at the moderate temperature (10–20°C) was visibly higher than that at the high temperature (20–30°C) and CWs with AgNPs addition had no appreciable differences compared with the control. High-throughput sequencing results indicated that the microbial richness, diversity and composition of CWs were distinctly affected with the extension of exposure time at different AgNPs levels. However, the phosphorus removal performance of CWs did not decline with the decrease of polyphosphate accumulating organisms (PAOs), which also confirmed that adsorption precipitation was the main way of phosphorus removal in CWs. The study suggested that AgNPs and phosphorus could be removed synergistically in the coexistence system. This work has some reference for evaluating the influences of AgNPs on the phosphorus removal and the interrelation between them in CWs.

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Introduction

Nanotechnology has developed rapidly in recent years, and artificial nanomaterials have been widely produced and applied due to their superior physical and chemical properties, which are extensively used in the electric, agriculture, biomedicine, new energy, daily necessities fields and so on (Hussein, 2015; Singh et al., 2015; Chan et al., 2017; Contreras et al., 2017). Because of a series of excellent properties and antibacterial properties (Baker et al., 2005; Li...
et al., 2008), silver nanoparticles (AgNPs) have become one of the most widely used and fastest growing nanoparticles (Nowack et al., 2011; Stensberg et al., 2011; Vance et al., 2015). AgNPs are inevitably released into the natural environment in the production, transportation, and utilization of the products. Some surveys reported that residential and industrial discharges were the main pathways of AgNPs entering into sewage treatment systems (Boxall et al., 2007; Kim et al., 2010). In the field of water treatment, the adverse impacts of nanoparticles on biological sewage treatment systems have become an international research focus.

Phosphorus is the primary element causing eutrophication of waters, and has become a worldwide problem in water quality (Zheng et al., 2014). In recent years, there have been many studies involving the impacts of nanoparticles on the phosphorus removal performance of wastewater biological treatment systems. Zheng et al. (2011) suggested ZnO NPs dosing with high levels (10 and 50 mg/L) could induce the loss of normal phosphorus removal in activated sludge system. Wang et al. (2017) found that the soluble orthophosphate (SOP) removal showed an evident decrease at CuO NPs (>2 mg/L) in sequencing batch reactor (SBR), however, the removal rate kept a relatively stable value with the concentration of CuO NPs increasing from 2 to 60 mg/L. And this result was consistent with the variation tendencies of exopolyphosphatase (PFK) and polyphosphate kinase (PFK). Li et al. (2017) indicated that TiO₂ NPs impacted the phosphorus removals of activated sludge with the phosphorus removal efficiency gradually decreasing at 0.5 mg/L TiO₂ NPs and increasing at 10–60 mg/L TiO₂ NPs, which had been associated with the phosphorus release and uptake rates and corresponding microbial enzymatic activities.

Chen et al. (2012) investigated the influences of AgNPs and Ag⁺ (0–5 mg/L) on wastewater enhanced biological phosphorus removal (EBPR), and found that regardless of the concentrations of AgNPs, the P-removal efficiency was maintained at 99%. When concentration of Ag⁺ was higher than 1 mg/L, however, the removal rate of phosphorus was decreased significantly. In addition, Chen et al. (2013) reported that the phosphorus removal rate was reduced by a sudden increase in Ag⁺ concentration and recovered gradually after a long period of cultivation of the EBPR. Zhang et al. (2016) illustrated that TiO₂ NPs impacted the phosphorus removals of activated sludge with the phosphorus removal efficiency gradually decreasing at 0.5 mg/L TiO₂ NPs and increasing at 10–60 mg/L TiO₂ NPs, which was high 10 cm. The CWs were composed of four layers: 10 cm high of 30 mm coarse zeolite particles at the upper layer; 20 cm high of 20 mm medium gravel particles at the middle layer; 15 cm high of 5 mm small gravel particles at the upper layer; 20 cm high of soil at surface layer. Iris pseudacorus was selected as wetland plant with a density of 20–100 plants per square meter. The CWs had been fed with tap water for several months to promote plants growth and adaption to wetland conditions.

As an efficient and stable ecological treatment technology for sewage, constructed wetlands (CWs) are also faced with the challenge of the emerging pollutants of metal nanoparticles (Keller and Lazareva, 2014). On the one hand, constructed wetlands can effectively remove silver nanoparticles in influent (Geisler-Lee et al., 2014; Quah et al., 2015; Auvinen et al., 2016, 2017). But at the same time, the ecotoxicity of AgNPs affects the removal performance of nutrients in CWs to some extent (Huang et al., 2017, 2018; Cao et al., 2018). In previous studies, we found that total phosphorus (TP) removal were obviously affected by AgNPs treatment (50 and 200 µg/L), with the removal efficiency reducing from about 70% to about 50% within 90 days (Huang et al., 2017). On this basis, we conducted a more than one year (450 days) monitoring of the CWs exposure to AgNPs, with the purpose of further exploring the relationship of AgNPs and phosphorus in the coexisting system including the influences of persistent AgNPs treatment on phosphorus removal performance and dynamic changes of CWs microenvironment.

The objectives of the present study were (1) to evaluate the long-term effects of different AgNPs levels on the TP removal and the relationship of AgNPs and phosphorus removal in soil layer; (2) to analyze the enzymatic activity of phosphatase in CWs; (3) to clarify the changes of microbial community structure of CWs after long-term exposure to AgNPs as well as phosphorus removal related microorganisms.

1. Materials and methods

1.1. Preparation of silver nanoparticles suspension

In this study, the monomer suspension of silver nanoparticles was purchased from Shanghai Huzheng Nano Technology Co., Ltd., Shanghai, China. Using physical chemistry methodology, the monomer AgNPs suspension was coated by polyvinyl pyrrolidone (PVP). AgNPs suspension was prepared in accordance with previous methods (Huang et al., 2017). The shape and size distribution of AgNPs were measured by Transmission Electron Microscope (TEM). From Appendix A Fig. S1, AgNPs was well dispersed and spherical with 10–40 nm in diameter.

1.2. Set-up and operation of the CWs

Three vertical flow constructed wetlands were constructed with PVC columns in the laboratory of Southeast University (Appendix A Fig. S2). The dimension of them was 30 cm in diameter and 80 cm in height and the bottom was conical, which was high 10 cm. The CWs were composed of four layers: 10 cm high of 30–40 mm coarse zeolite particles at the bottom; 15 cm high of 10–20 mm medium gravel particles at the middle layer; 15 cm high of 5–8 mm small gravel particles at the upper layer; 20 cm high of soil at surface layer. Iris pseudacorus was selected as wetland plant with a density of 20–100 plants per square meter. The CWs had been fed with tap water for several months to promote plants growth and adaption to wetland conditions.

The hydraulic retention times (HRTs) were 34 hr and the hydraulic loading (HLR) was controlled at 0.1 m/day by the peristaltic pump (BT100-1L, Longer Pump Co., Ltd., China). The continuous monitoring of CWs were 480 days between March 2016 and June 2017. In the process of the study, the operation of each CW was stable.

1.3. Synthetic wastewater

Synthetic wastewater was prepared for experiment (containing COD = 200 mg/L, TN = 20 mg/L, TP = 3 mg/L). The CWs were fed with synthetic wastewater composed of (mg/L): C₆H₁₂O₆, 200; (NH₄)₂SO₄, 66; CO(NH₂)₂, 12.85; KH₂PO₄, 13.16; and 5 mL
trace-element solution (mg/L) containing MgSO₄·7H₂O, 50; FeSO₄·7H₂O, 4.5; ZnSO₄·7H₂O, 0.13; Na₂MoO₄·2H₂O, 0.03; H₃BO₃, 0.025; CuSO₄·5H₂O, 0.03. All reagents were prepared in deionized water.

There was a 30-day steady operated period prior to AgNPs dosing in the influent. Synthetic wastewater with AgNPs concentrations of 0, 50 and 200 μg/L flowed into the CW1-3, respectively.

1.4. Analytical methods

Water quality parameters were determined according to Standard Methods (APHA, 2005). The silver concentrations were measured by inductively coupled plasma-mass spectrometry (ICP-MS) (1260/7700X, Agilent Technologies Inc., Santa Clara, California, USA). The activity of acid-phosphatase (ACP) was measured using a spectrophotometric method with p-nitrophenyl phosphate (pNPP) as a substrate. Operation methods were carried out according to the methods of Acikel and Ersan (2010). The p-nitrophenol released was measured at 410 nm in spectrophotometer. One unit of phosphatase activity was defined as the amount of enzyme solution liberating 1 mol p-nitrophenol per min at pH 4.8 and 37°C.

1.5. DNA extraction and high-throughput sequencing

Soil samples were collected from the soil layer of CWs on the Day 290 (January 2017) and Day 448 (June 2017), which were taken from the upper soil layer (0–5 cm) and the lower soil layer (15–20 cm). Then the soil samples collected twice were mixed evenly and immediately stored at −20°C until use. Each DNA sample was extracted by using Powersoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, USA) in accordance with manufacturer’s instructions. The V4 regions of the bacteria 16S rDNA genes were amplified by PCR (98°C for 2 min, followed by 27 cycles at 98°C for 15 sec, 50°C for 30 sec, and 72°C for 30 sec and a final extension at 72°C for 5 min) using primers 520F (5′-AYTGGGTYDTAAAGNG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′). An Illumina Miseq (Shanghai Personalbio Technology Co., Ltd.) was applied for sequencing the genes of collected samples. In MOTHUR (http://www.mothur.org/), Shannon diversity index, Chao 1 index, Simpson index for each sample were analyzed. The similarity and difference of soil samples at different AgNPs concentrations were described by shared and unique Venn diagram of OTUs.

1.6. Statistical analysis

All tests assays were conducted in triplicate and the results were expressed as mean ± standard deviation. All data plots represented the average of at least three independent experiments, and the error bar represented a confidence interval of 95%.

2. Results and discussions

2.1. Impacts of AgNPs on the TP removal

For the purpose of studying the effect of AgNPs on phosphorus removal in CWs, we measured the concentration of TP in effluent at different stages and obtained the removal rate of TP from CWs. As shown in Fig. 1, compared with the control CW1 (0 μg/L), total phosphorus removal efficiency of CW2 (50 μg/L) and CW3 (200 μg/L) declined in the short-term exposure. However, the inhibitory effects gradually disappeared during the partial medium-term and long-term treatment. Specifically, the short-term exposure (0–90 days) of AgNPs evidently inhibited the phosphorus removal with TP removal efficiency from 75% (CW1–3) reducing to 55.70% ± 5.46% in CW2 (50 μg/L) and 50.62% ± 4.41% in CW3 (200 μg/L). In addition, the degree of inhibition was positive correlated with AgNPs concentration, which was consistent with previous studies (Huang et al., 2017). At medium-term exposure stage (210–300 days), TP removal efficiency fluctuated slightly, and related environmental factors, such as low temperature (November 2016 to January 2017, temperatures measuring between 11.0 and 15.0°C), were not excluded. After day 330, TP removal rates gradually recovered and maintained stability with removal efficiency 77.30% ± 8.06% in CW1, 77.15% ± 6.30% in CW2 and 80.39% ± 6.11% in CW3, respectively. The present results demonstrated that persistent exposure of AgNPs (0–200 μg/L) had no significant negative influence on the performance of phosphorus removal in CWs. This can also be confirmed by the effluent concentration of SOF from CW systems (Appendix A Fig. S3). It is noteworthy that CW3 with AgNPs (200 μg/L) addition performed more desirable in phosphorus removal, especially in the long-term exposure. Some explanations have been given in other reports that the presence of nanoparticles in the influent, which stimulated the corresponding microbial enzymatic activities, could promote the phosphorus removal to an extent (Li et al., 2017; Ma et al., 2017). Other studies have found that phosphorus removal performances of WWTP (waste water treatment plant) were recoverable after a chronic exposure of nanoparticles, such as TiO₂ NPs (0–60 mg/L) in activated sludge reactors (Li et al., 2017) and Cu NPs (0–50 mg/L) in biological phosphorus removal systems (Chen et al., 2015). As far as the studies of AgNPs were concerned, Chen et al. (2012) investigated the impacts of AgNPs and Ag⁺ (0–5 mg/L) on enhanced biological phosphorus removal wastewater treatment, and they found that the phosphorus removal efficiency was maintained at 99% not affected by the concentration levels of AgNPs but decreased obviously at Ag⁺ concentration levels of 1 mg/L. It could be inferred that the stronger toxic effects of a small amount of dissolved silver ions inhibited phosphorus removal in a short-term exposure of AgNPs (Kittler et al., 2010; Chen et al., 2013). However, with the extension of exposure time, the humus substances would accumulate in the soil near the root zones, which alleviated the biotoxicity of AgNPs by inhibiting the dissolution of Ag⁺ from AgNPs (Sujin et al., 2013; Zhang et al., 2016). Another argument was that AgNPs in wastewater treatment systems could be transformed into Ag₂S or Ag–sulphhydryl complexes, thereby reducing toxicity (Levard et al., 2012; Yuan et al., 2015). These reports could reasonably explain the decrease of AgNPs ecological toxicity for a long-term exposure. Whether the CWs can effectively remove AgNPs while ensuring a favorable phosphorus removal? In other words, the competitive or cooperative relationship of AgNPs and phosphorus removal in CWs needs further research.
2.2. The relationship of silver nanoparticles and phosphorus in soil layer

The sorption and immobilization of phosphorus were known as the major phosphorus removal pathways of soil layer in wetlands. Generally, phosphorus binds to calcium and aluminum in soil to form poor solubility phosphate, or it is immobilized by minerals such as iron and aluminum oxides. Meanwhile, the soils are complex matrices containing solid and liquid phases, which consist of abundant minerals, organic matter, natural colloids and particulate matter (Torrent et al., 2016). As a result, AgNPs negatively charged can easily interact with these components of the soil when it goes into the CWs with sewage. These series of interaction processes including adsorption, aggregation/agglomeration and sedimentation, which make the soil layer act as a large sink of AgNPs. Therefore, the competitive or cooperative removal relationship between phosphorus and AgNPs in soil layer is worth further exploration. Three time points were selected during the short-term and long-term exposure of AgNPs to analyze the content of TP and Ag in the effluent of the soil layer and the removal efficiencies of TP and AgNPs in soil was obtained. As shown in Fig. 2, from 0 to day 90, the removal rate of AgNPs and TP presented an opposite trend. That is, the removal rate of TP decreased while the removal rate of AgNPs increased in both CW2 and CW3. These results implied that the removal of AgNPs and phosphorus in soil layer showed a competitive relationship during the initial exposure to AgNPs. On the one hand, the stability of soil colloids and NPs could be decreased because of the presence of abundant cations in synthetic wastewater thus facilitating aggregation (Klitzke et al., 2015; Torrent et al., 2016). On the other hand, the weakly charged or even positively charged sites of clay-sized mineral surface are preferential attachment sites for negatively charged nanoparticles (Cornelis et al., 2012). Thus, AgNPs may compete with phosphate ions for binding sites on the soil particles, which leads to decreasing the amount of phosphorus adsorbed by soil layer (Fig. 3). During day 360–450, the trend of AgNPs removal rate was consistent with TP, and there was a synergistic removal relationship between them. Moreover, the silver and phosphorus removal efficiencies of soil layer were obviously higher than that at the early stage of AgNPs exposure. With the prolongation of CWs running time, the organic matter in soil increased. By complexing with the soluble organic compounds or forming silver sulfide precipitations, Ag ions adsorbed to soil particle surface were decreased (Benoit et al., 2013), which made soil P-sorption occupy more binding sites. In addition, the enhancement of plant uptake has also played a rather important role in contaminants including phosphorus and AgNPs removal in soil layer. Therefore, a cooperative removal was observed between phosphorus and AgNPs in CWs at the later operation stage (Fig. 3).

2.3. Impacts of AgNPs on the enzyme activity of phosphatase

As shown in Fig. 4, the activity of acid-phosphatase in soil layer of three CWs at the moderate temperature (10–20°C) was visibly higher than that at the high temperature (20–30°C). Although some previous studies had shown that a certain range of temperature rising could increase the soil phosphatase activities accordingly (Sardans et al., 2007; Gong et al., 2015; Ge et al., 2017), there were researches demonstrated that 15°C was the optimal temperature within 5–25°C for the development of microorganisms in soil and the highest activity of acid-phosphatase was observed at 15°C (Borowik
and Wyszkowska, 2016). Being consistent with other results of reaction rate Q10 values reduced at elevated temperature (Tjoelker et al., 2001; Razavi et al., 2015), Razavi et al. (2016) observed that phosphatase Vmax-Q10 was highest between 10 and 20°C and a decrease thereafter, suggesting that phosphatase could maintain high catalytic efficiency at the moderate temperature.

Secondly, it could be found that AgNPs within the concentration range of 0–200 μg/L had no appreciable difference on the enzyme activity of phosphatase. Specifically, the phosphatase activity of CW2 and CW3 with AgNPs added into the influent had nearly no difference with the control CW1 (without AgNPs) at the moderate temperature while a little bit higher than that at high temperature. Hansch and Emmerling (2010) evaluated the effects of AgNPs on enzyme activities and found that it didn’t inhibit the enzyme activity of phosphatase when the concentration of AgNPs was 0.32 μg/g. Moreover, Peyrot et al. (2014) found that AgNPs (1–100 μg Ag/kg) produced significant negative effects on the soil enzyme activities tested including phosphomonoesterase, arylsulfatase, β-glucosidase, and leucine-aminopeptidase, however, phosphomonoesterase observed to be the least affected enzyme. A number of studies revealed acid phosphomonoesterase was known for having a poor sensitivity in the presence of Cd (Renella et al., 2004), Cr,

Figure 2 – Removal efficiency of TP and AgNPs in soil layer. Error bars represent standard deviations of measurements within 30 days (n = 7, water samples were collected every 4 days). AgNPs removal efficiency = (C_{in} - C_{out, soil})/C_{in} × 100%, C_{in} (μg/L) is the AgNPs concentrations in influent of CW and C_{out} (μg/L) is the AgNPs concentrations in effluent of soil layer, respectively.

Figure 3 – Potential relationships between AgNPs and phosphorus in soil layer.
Ni, and Cu (Warman and Munroe, 2010) even when subjected to relatively high contamination levels. Warman and Munroe (2010) also reported that there were no significant relationships between acid phosphatase and soil Pb2+ loadings from 0 to 2400 mg/kg in two kinds of soils. High contamination of metal in soil had even been found to activate the enzymes extracellular soil enzyme such as phosphatase activities, which were notably high at a site with the highest heavy metal loads, research had even found (Hagmann et al., 2015). On one hand, excepted for the enzyme itself, the physico-chemical properties of the soils such as pH, oxidation reduction potential, and content of minerals and so on might play a role in determining the effect of the NPs on the enzymatic activity (Josko et al., 2014; Cele and Maboeta, 2016). On the other hand, enzyme as a kind of protein, needed a certain amount of metal ions as an auxiliary group, which promoted the coordination bonding between the center of enzyme and substrate and changed the surface charge of enzyme protein to enhance the enzyme activities (He et al., 2018). Therefore, high concentration (≥ 200 µg/L AgNPs) of the 290th day soil samples. In addition, there were 39,527, 43,998, and 46,947 high-quality sequences obtained at 0, 50, and 200 µg/L AgNPs of the 290th day soil samples. The OTUs number of CW1–3 (0, 50, and 200 µg/L AgNPs) were 1649, 1778, and 2087 on the 290th day as well as 1630, 1658, and 2342 on the 448th day. At different AgNPs levels, the Good’s coverage was always above 0.95, indicating that the sequence libraries could cover the microbial species diversity of soil samples.

For soil samples collected on the 290th day, the total OTUs numbers could be estimated as 1945.29 (CW1), 2302.22 (CW2), and 2154.96 (CW3) by Chao 1 estimator with infinite sampling. This index could indicate that the 50 µg/L AgNPs treatment increased the richness most noticeably, but 200 µg/L AgNPs promoted the richness less evidently. The species richness and distribution of microbiome can be described by Shannon diversity index. Among the three communities, CW2 (Shannon = 8.27) had the highest diversity, while CW3 (7.41) was slightly lower than CW1 (7.97). With regard to soil samples collected on the 448th day, the number of OTUs could be calculated as 1631.00 (CW1), 1667.00 (CW2), and 2344.16 (CW3) by Chao 1 indexes. With increasing contact time, the richness of CW1and CW2 (0, 50 µg/L AgNPs) declined while the richness of CW3 (200 µg/L AgNPs) raised obviously. From the standpoint of Shannon index, CW3 (8.92) had the highest diversity and evenness while the control (0 µg/L) with the lowest index of 6.97. In consequence, it could be deduced from the results that high concentration (200 µg/L) AgNPs with a long-term exposure could promote the richness and evenness of microbial communities in CWs.

For the purpose of evaluating the distinction and similarity of microbiome in the soil of CWs at different AgNPs levels, a Venn diagram was constructed containing common and unique OTUs (Fig. 5a, Fig. 5b). Total OTUs observed on Day 290 was 2743 in the soil at 0, 50, and 200 µg/L AgNPs, and 898 OTUs embracing 32.74% of the total OTUs were shared by them. The dominant phyla of the shared OTUs in CW1–3 soil samples were Proteobacteria (44.60%), Bacteroidetes (14.62%), Verrucomicrobia (7.45%), Acidobacteria (6.99%), OD1 (6.58%), Fusobacteria (2.88%), Chloroflexi (2.87%), Actinobacteria (2.31%), Spirochaetes (2.14%), Nitrospirae (1.41%), Firmicutes (1.21%), Parvarchaeota (1.12%). CW2 and CW3 had the most common OTUs (1356, 49.43% of total), while CW1 and CW2 shared the least ones (1126, 41.05% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total). OTUs unique to each samples were 437 (1126, 31.72% of total).

2.4. Change of microbial community under AgNPs exposure

2.4.1. Richness and diversity of microbial community
Table 1 summarizes the richness and diversity indices of microbial community at different AgNPs concentrations and different exposure times. The high-quality sequences at 30,011, 32,235, and 33,290 were obtained at 0, 50, and 200 µg/L AgNPs of the 290th day soil samples. In addition, there were 39,527, 43,998, and 46,947 high-quality sequences obtained at 0, 50, and 200 µg/L AgNPs of the 448th day soil samples. The OTUs number of CW1–3 (0, 50, and 200 µg/L AgNPs) were 1649, 1778, and 2087 on the 290th day as well as 1630, 1658, and 2342 on the 448th day. At different AgNPs levels, the Good’s coverage was always above 0.95, indicating that the sequence libraries could cover the microbial species diversity of soil samples.

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Correspondingly, total OTUs observed on Day 448 was 3873 of the soil samples, but only 522 OTUs accounting for 13.48% of the total OTUs were shared by them. Most shared OTUs belonged to Proteobacteria (37.15%), Chlorobi (26.21%) and Bacteroidetes (8.64%). CW1 and CW2 had the most common OTUs (792, 20.45% of total), while CW1 and CW3 shared the least ones (727, 18.77% of total). For each community of CWs, the OTUs unique were 634, 625, and 1367, respectively, accounting for 67.80% of the total observed OTUs. Moreover, the OTUs unique of CW3 (1367) was the maximum, indicating that high concentration (200 μg/L) AgNPs exposure could markedly alter species diversity of the microbiome over time.

2.4.2. Microbial community shift on the phylum level
In order to further characterize the phylogenetic differences and community successions of CWs at different AgNPs levels, the taxonomic compositions of microbiome were compared at phylum level on Day 290 and Day 448.

In total, 43 phyla were classified under the persistent exposure of AgNPs for 290 days and detail composition of 16 phyla (occupied >0.5%) of each sample were presented in Fig. 6a-1. The results showed that the relative abundance of the dominant phyla Proteobacteria, Bacteroidetes, Acidobacteria, Verrucomicrobia, OD1, Chloroflexi, Actinobacteria, Spirochaetes and Nitrospira were 39.37%–49.55%, 12.98%–17.32%, 4.80%–10.47%, 5.80%–8.69%, 4.19%–10.59%, 2.04%–3.77%, 1.26%–3.74% and 1.03%–2.29% in CW1-3, respectively. There were 60 phyla been classified during the constant exposure of AgNPs for 448 days and specific members of 16 phyla (occupied >1%) of each sample were presented. As shown in Fig. 6a-2, the relative dominant bacterial phyla Proteobacteria, Chlorobi, Bacteroidetes, Chloroflexi, Spirochaetes, Acidobacteria, Nitrospira, and Firmicutes were 30.86%–43.69%, 7.90%–41.09%, 5.70%–12.72%, 3.44%–6.15%, 2.31%–7.75%, 1.47%–6.92%, 1.20%–1.71% and 1.03%–2.30% in three soil samples. On the whole, Proteobacteria and Bacteroidetes were always two dominant phyla in CW systems during the overall period of AgNPs exposure, which was similar as other researches such as enhanced biological phosphorus removal system under the stress of ZnO nanoparticles (Hu et al., 2017) and SBR activated sludge system at AgNPs exposure (Zhang et al., 2016). Besides, some studies also pointed out that Proteobacteria and Bacteroidetes were the dominant phyla in wastewater treatment plant (WWTP) (Chu et al., 2015; Cao et al., 2017). Interestingly, with the increase of AgNPs concentration (0–200 μg/L), the abundance of Proteobacteria decreased in the previous stage (0–290 days), while the abundance promoted in the latter stage (290–448 days). Anyway, the results presented that Proteobacteria was the most major phylum in the soil samples during the overall period, indicating that Proteobacteria was the main phylum in the P removal process. Besides, the high concentration of AgNPs (200 μg/L) could obviously stimulate the abundance of Bacteroidetes, which could hydrolyze macromolecule organic matter into small molecule, such as propionic acid, acetic acid, and release methane under the anaerobic condition as a common microorganism in wetlands (Dong and Reddy, 2010). It was worth noting that Chlorobi gradually became dominant communities during the Day 290 to Day 448, but the abundance of it was extremely small (<1%) before the Day 290. Chlorobi was a class of specialized photosynthetic

Fig. 5 – Venn of the bacterial communities (a) in the 290th day, and (b) in the 448th day soil samples of three CWs with different AgNPs levels on OTUs at 3% distance. The shared OTUs were analyzed at phylum level and the number in figure represents the OTUs in the corresponding area.
bacteria, which were suitable for survival under conditions of anoxic by using reduced sulfur compounds as electronic donors to provide energy and reducing iron redox proteins to achieve carbon sequestration, biosynthesis and cell growth (Bryant and Frigaard, 2006; Frigaard and Dahl, 2009). It was implied that the oxygen supply in the CWs was inadequate with the extension of operation time.

2.4.3. Microbial community shift at the class level
The relative microbial community abundance on the class level was further analyzed. Among the tested soil samples on Day 290, the top 20 abundant class were selected (Fig. 6b-1). The majority class in the Day 290 soil samples belonged to α-, β-, γ-, δ- and ε-proteobacteria, Bacteroidia, Holophagae, Fusobacteria, Cytophagia, ZB2, Anaerolineae, Spirochaetes,
Actinobacteria and Nitrospira at different AgNPs concentrations. Results showed that Proteobacteria, except α-proteobacteria, was changed significantly by the exposure to AgNPs. The β-proteobacteria, to which many Phosphorus Accumulating Organisms (PAOs) might belong (Kong et al., 2007; Stockholm-Bjerregaard et al., 2017), were inhibited by AgNPs obviously (decreased from 35.63% in control to 18.28% at 200 μg/L). As the concentration of AgNPs from 0 to 50 μg/L, the relative abundance of γ-proteobacteria increased, but decreased at 200 μg/L AgNPs. Previous studies identified that some members of Halomonadaceae (Nguyen et al., 2012) and Pseudomonadaceae (Guenther et al., 2009) belong to γ-proteobacteria were PAOs, while the majority members of γ-proteobacteria were classed as Glycogen Accumulating Organisms (GAOs) (McIlroy et al., 2017; Hu et al., 2017). Furthermore, the relative abundance of δ- and ε-proteobacteria increased with AgNPs concentration.

As shown in Fig. 6b-2, the dominant class in the Day 448 soil samples belonged to Chlorobia, α-, β-, γ-, δ- and ε-proteobacteria, Bacteroidia, Holophagae, Sphingobacteria, Anaerolineae, Spirochaetes, Nitrospira, Clostridia, Bacteroidetes_vadinHA17 and Bacteroidia at different AgNPs concentrations. It was also worth pointing out that the abundance of Chlorobia became dominant class with the extension of operation time of the constructed wetlands. This phenomenon was consistent with the change of microbial community at phyla level. As far as Proteobacteria were concerned, the abundance ratio of δ- and ε-proteobacteria increased with the AgNPs concentration, which was the same as the results of Day 290. On the contrary, the abundance ratio of γ-proteobacteria decreased with AgNPs levels from 0 to 50 μg/L, and then increased at a high concentration AgNPs (200 μg/L). β-proteobacteria were stimulated by AgNPs at 50 μg/L but inhibited at 200 μg/L AgNPs. Overall, from the time of vertical perspective, the relative bacterial abundance of γ-proteobacteria increased and β-proteobacteria decreased from Day 290 to Day 448 at different AgNPs concentrations.

### 2.4.4. Microbial community shift at the genus level

More critical information for the functional bacterial community was provided based on the genus classification. As shown in Fig. 6c-1 and Fig. 6c-2, the top 17 abundant genera came from each sample were selected and their abundances were compared at different AgNPs concentrations.

Geothrix, Geobacter, Dechloromonas, Treponema and Pseudomonas dominated in the soil samples on Day 290. Dechloromonas, belonging to Rhodocyclaceae under the class of β-proteobacteria, was a genus capable of reducing perchlorate (Achenbach et al., 2001), accumulating polyphosphate and PHA, and taking up carbon under anaerobic conditions (Kong et al., 2007). The relative abundance of Dechloromonas was 3.75% in CW2 (50 μg/L) while decreased to 4.58% in CW3 (200 μg/L). Considering most members of the genus, Dechloromonas have been shown to behave according to the PAO phenotype, some previous researches reported it as PAOs in enhanced biological phosphorus removal (EBPR) systems (Liu et al., 2005; Kong et al., 2007; Guenther et al., 2009). In addition, small proportion of this genus have also been shown potentially behave as their GAO competitors in an aerobic EBPR system without contributing to P removal (Ahn et al., 2007). Pseudomonas, belonged to γ-proteobacteria, which had a strong ability to absorb phosphorus from sewage and stored it in cells in the form of polyphosphate (Lotter and Murphy, 1985; Suresh et al., 1985). Members of the genus Pseudomonas were an efficient microorganism for phosphorus removal and been identified as PAOs in biological systems by applying a polyphosphate staining method with fluorescence activated cell sorting (Guenther et al., 2009). As the concentration of AgNPs increased from 0 to 50 μg/L, the genus Pseudomonas decreased from 1.08% to 0.77%, but which was not changed as the concentration of AgNPs increased from 50 to 200 μg/L.

With the process of AgNPs exposure from Day 290 to Day 448, microbial community shift at the genus level was distinctly. Chlorobaculum, Halothiobacillus, Sulforhodanella, Sulfuricurvum and Geobacter became the dominant genus. Chlorobaculum was a genus capable of oxidizing sulphide and sulphur to produce sulphate as final product (Rodriguez et al., 2011). Halothiobacillus grew chemolithoautotrophically under salt environment by the oxidation of reduced sulfur compounds (Sievert et al., 2000; Wood et al., 2005). Geobacter was a typical dissimilatory metal-reducing bacteria, which were widely distributed in organic matter or heavy metal polluted environments and some groundwater sediments (Coates et al., 1996; Roling et al., 2001; Lovley et al., 2004). The abundance of Geobacter increased from 1.26% to 5.55% with the AgNPs concentration increasing from 0 to 200 μg/L. Further, the relative abundance of PAOs, Dechloromonas and Pseudomonas, decreased significantly (<0.1%) at different AgNPs concentration on Day 448, indicating that AgNPs could generate a certain degree of inhibitory effect on typical phosphorus removal bacteria.

From the whole shift of microbial community, it could be found that the chronic exposure of AgNPs had transformed the diversity and structure of microbiologic population to some extent, and also influenced some typical phosphorus-removing functional bacteria obviously. However, the effects of microbial community changes on the long-term phosphorus removal efficiency of CWs were not significant. It was speculated that PAOs couldn’t fully exploit the advantages to uptake phosphorus in aerobic environment due to the substrates environment persistently under anoxic or anaerobic condition, which greatly limited the functions of microorganisms on phosphorus removal in CWs. And again, it was confirmed that CWs was a system of interaction between substrate, microorganisms and plants (Gray et al., 2000; Stottmeister et al., 2003), and the process of P-sorption was the main pathway to remove phosphorus from CWs (Vymazal, 2007).

### 3. Conclusion

In the present study, performance of phosphorus removal of CWs was evaluated under the long-term AgNPs exposure. The TP removal efficiency was evidently inhibited during the short-term exposure of AgNPs, but gradually recovered after a long-term exposure. Moreover, in the initial AgNPs exposure phase, there was a competition between Ag and P removal in soil layer, whereas synergistic effect emerged in the later phase. The enzymatic activity of acid-phosphatase in soil was more related to the temperature, while had not obvious correlation with AgNPs concentration. The microbial richness,
diversity and composition of CWs were distinctly affected with the extension of exposure time at different AgNPs concentrations. On the whole, AgNPs are unlikely to affect the efficient functioning of CW systems in terms of phosphorus removal because Ag and P could be co-removed through soil adsorption. However, AgNPs accumulate in the soil layer may negatively affect key functional microorganisms. Thus, the potential threats of AgNPs exposure to ecological environment should not be ignored.

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Appendix A. Supplementary data

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References


