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Research Paper

Contradictory effects of silver nanoparticles on activated sludge wastewater treatment

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HIGHLIGHTS

- Ag-NPs, especially freshly prepared, can have positive effects.
- Ag-NPs can help to maintain microbial community diversity in activated sludge.
- Improved sludge settleability can be important in the positive effects observed.
- The hormesis model may need to be considered for the toxicology of Ag-NPs.

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ABSTRACT

Increased amount of nano-silver will be released into domestic and industrial waste streams due to its extensive application. However, great controversy still exists on the effects of silver nanoparticle (Ag-NP) on biological wastewater treatment processes and a toxicology model has not been built yet. Four sequencing batch reactors with activated sludge has been run for over three months with different silver species at a concentration of 1 mg Ag/L in influent. Both freshly prepared Ag-NPs and aged Ag-NPs were tested with released silver ion as control. Results in this study showed that Ag-NPs, especially freshly prepared Ag-NPs, can help to maintain or even increase the diversity of microbial community in activated sludge and the biomass concentration even under long-term treatment. It indicates that the hormesis model need to be considered for the toxicology of Ag-NPs.

1. Introduction

Nano-silver is inevitably released into domestic and industrial waste streams as it is one of the most commonly used nano materials in consumer products [1]. Considerable attention has been paid to the potential adverse effects on biological wastewater treatment system (BWTS) due to the antimicrobial properties of silver nanoparticles (Ag-NPs). A general conclusion can be made from previous research that the effects of Ag-NPs depend on the dose and time period applied as well as the property of Ag-NPs and the system Ag-NPs are applied to. However, great controversy still exists on how each of these parameters affects the impacts of Ag-NPs, and a sophisticated toxicology model has not been built at all. Previous research covers only a tip of the iceberg of all possible combination of these parameters. Not to mention that the mechanisms behind the phenomena are poorly understood.

Higher concentration of Ag-NPs often results in more significant adverse effects [2–5]. Hormetic effects under sublethal concentration have been reported occasionally but stayed as a marginalized concept [6–10]. Properties of Ag-NPs that affect its toxicity include nanoparticle size, shape and coating. Smaller Ag-NPs tend to be more toxic [11–13]. Spherical Ag-NPs and polyvinylpyrrolidone (PVP) coating tend to have weaker bactericidal action [14,15]. However, the effects of shape and coating have not been well-studied yet. Great controversy also exists on if the toxicity of Ag-NPs come from the released Ag^+ ion or the nanoparticle form itself [3,7,14,16–18].

Properties of the BWTS are even more complicated. The effects of Ag-NPs on activated sludge and biofilm in BWTS have been studied previously [19]. Ordered from the most resistant to Ag-NPs to the least, the microbial communities in BWTS include biofilm/activated sludge, planktonic mixed culture and pure culture of single strains

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[2,9,20,21]. Potential ligands in BWTS can bind with Ag-NPs or released Ag^+ ions and lower the dissolution of Ag-NPs and their bactericidal effects [22–24], although some anions may accelerate Ag-NP dissolution [25]. These ligands range from organic matter such as dissolved organic carbon to inorganic ions such as chloride and sulfide. It has been reported that ammonia oxidizing bacteria (AOB) are more vulnerable towards Ag-NPs treatment, as compared to nitrite oxidizing bacteria (NOB) and organic oxidation heterotrophs [20,26–28]. More recent studies tend to focus on long-term effects of Ag-NPs under conditions mimicking the real-world conditions in BWTS [21,29,30]. Acute inhibition is often observed at the beginning of Ag-NP addition, but the system usually recovers in the long term [4,5]. For instance, in a membrane bioreactor activated sludge system with 0.1 mg/L Ag-NPs in the reactor influent, the silver resistance gene (*silE*) increased at the beginning of the Ag-NPs addition and then decreased to the initial level; and the reactor performance was not significantly affected by Ag-NPs [31]. In a separate study, with the addition of 1 and 5 mg/L Ag-NPs in bioreactors, phosphorus removal decreased and the microbial community changed at the beginning of the study but then stabilized with persistent exposure [30]. The adverse effects of Ag-NPs are minimal especially when sulfidation plays an important role in most of the BWTSs [32–37]. A toxicological model to estimate the effects of Ag-NPs is beginning to take shape. This raises the question: should the hormesis model be considered here?

This study examines the response of the microbial community to a potentially "least-toxic" combination, which is the case in most of our current BWTSs in practical operation: low dose, long-term, spherical Ag-NPs with PVP coating in activated sludge bioreactors fed with synthetic municipal wastewater. No significant effects were seen on pollutants removal. However, interestingly, Ag-NPs helped to maintain the microbial community diversity in the activated sludge. 16S rRNA gene based pyrosequencing was used to monitor the bacterial community and GeoChip was used to directly examine the functional diversity of the microbial community. Properties of the sludge, accumulation of silver species inside the sludge and characteristics of the Ag-NPs were examined to explain this phenomenon.

2. Material and methods

2.1. Reactor setup

Four sequencing batch reactors were operated for over three months. The total volume of the reactors was 1 L and the effective volume was 700 mL. The reactors were run on a 12 hour cycle (5 min of influent filling, 11 h of aeration, 30 min settling, 5 min effluent withdraw and 20 min idle). Hydraulic retention time was 24 h. Sludge was wasted through effluent withdraw by gravity. Solids retention time was monitored but not controlled. Solids retention time (SRT) was 17 days at the steady state for all four reactors. However, after the addition of silver species started, almost no sludge was wasted from the reactor with fresh Ag-NPs added, indicating that SRT was dramatically increased after the addition of fresh Ag-NPs. The reactor feed was prepared according to Alito and Gunsch [5] and contains an average COD of 450 mg/L and ammonia of 40 mg/L with pH adjusted to 7.3 ± 0.5.

2.2. Silver species addition

Self-dispersing silver nanopowder was purchased from SkySpring Nanomaterials, Inc. (Houston, USA). According to the Ag-NP product description, the particle size is less than 15 nm, and the particle composition is 25% silver (99.99% purity) and 75% polyvinylpyrrolidone (PVP), similar to Ag-NPs commonly used in commercial products. Fresh and aged Ag-NP suspensions were examined by transmission electron microscopy (TEM) according to the method described in previous studies [38]. Spherical aggregates of Ag-NPs were observed. The particle size and zeta potential of Ag-NPs were characterized using a Malvern Zetasizer Nano-ZS (Model: ZEN3600, Malvern Instruments Ltd, Worcestershire, UK). Ag^+ ion dissolution was characterized as described in Section 2.5. PVP and silver species addition started after the reactor reached steady state for over two weeks (27 days after start-up). PVP, aged Ag-NPs, fresh Ag-NPs and Ag^+ ion released from fresh Ag-NPs were added to each of the four reactors respectively. Aged and fresh Ag-NPs were added at a concentration of 1 mg Ag/L in influent. This concentration resulted in 0.5 mg Ag/L in the reactor, which falls within a representative range [39] while it was high enough to see significant effects based on previous tests (data not shwon). PVP was added to the control reactor at the concentration of 3 mg/L which is the same as in reactors with Ag-NP addtion. Aged Ag-NPs stock suspension was prepared when reactors were started up and kept at 4 °C in dark and was added into the influent tank and kept under room temperature in dark for one week. Fresh Ag-NPs suspension (3.5 g $Ag\,L^{-1}$) was prepared everyday and 0.1 mL suspension was spiked into the reactor during influent filling in each cycle, producing a concentration equalled to 1 mg Ag/L in influent. For the fourth reactor, to test Ag^+ ion released from fresh Ag-NPs, 0.1 mL of the freshly prepared Ag-NP suspension was added into a dialysis unit (Slide-A-Lyzer™ MINI Dialysis Device, 2 K MWCO, 0.1 mL, Thermo Scientific, USA) and the dialysis unit was put into the reactor during influent filling in each cycle and float in the activated sludge for 12 h before changing to a new one. Equal amount of PVP (3 mg $PVP\,L^{-1}$) was added into the control reactor. The tests of Ag-NP and PVP addition have been performed for over two months. Similar operation of reactors and Ag-NP addition were repeated for several times.

2.3. Reactor performance monitoring

Effluent quality was monitored in terms of COD and ammonium removal using Hach methods 8000 and 10205 [40]. SVI and MLSS were measured according to the standard methods [41]. Reaction kinetics of COD and ammonium removal and nitrate production was also performed using the substrate depletion method [42]. Mixed liquor samples were collected at 15, 30, 35, 60, 90, 120, 150, 180, 210, 240, 300, 480 and 660 min, centrifuged at 3000 g for 10 min at 4 °C, filtered (0.45 μm) and analyzed for COD, ammonium and nitrate. Nitrate was measured using ion chromatography (IC).

2.4. Microbial community analysis

Activated sludge samples were collected in duplicates and genomic DNA was extracted using a Powersoil® DNA Isolation Kit from MO BIO Laboratories, Inc. (Carlsbad, USA). DNA was analyzed with pyrosequencing and GeoChip.

Paired-end sequencing based on the 16S rRNA gene was performed at the Research and Testing Laboratory (Lubbock, TX, USA), using the Illumina MiSeq platform [43]. Primers 28F (5′-GAGTTTGATCNTGGCTCAG-3′) and 519R (5′-GTNTTACNGCGGCKGCTG-3′) were used, which covered V1–V3 hypervariable regions [44]. Chimeras and poor quality sequences were removed from the denoised sequence reads. The remaining sequences were clustered into operational taxonomic units (OTUs) with 0% divergence using USEARCH. Taxonomic information was assigned to OTUs based on a database of high quality sequences derived from the NCBI using a distributed. NET algorithm that utilizes BLASTN+ (Kraken BLAST, www.krakenblast.comwww.krakenblast.com). A principal coordinates analysis (PCoA) of micro-

bial community diversity was performed using the QIIME pipeline (http://qiime.org/http://qiime.org/) with the beta diversity metrics of weighted unifrac [45]. The canonical correspondence analysis (CCA) was carried out using the XLSTAT software version 2016 to evaluate the correlation between microbial communities and silver species addition. Ag-NP treatment was converted into four quantitative variables: silver accumulation, silver added as Ag^+ ion, silver added in the nanoparticle form, and PVP added.

DNA (1 μg) was labeled with Cy3 and hybridized to the GeoChip 5 microarray synthesized by NimbleGen (Madison, WI, USA) and processed as previously described by Lu et al. [46]. The signal-to-noise ratio threshold for a spot to be considered positive was ≥2 as described previously [47]. Detrended correspondence analysis (DCA) was performed based on sample scores. Hierarchical clustering analysis based on the Bray-Curtis dissimilarity indices was performed and a corresponding heatmap was built with the top 30 abundant functional genes in each sample.

2.5. Silver accumulation and release and nanoparticle characterization

Silver species accumulation and release was measured with inductively coupled plasma mass spectrometry (ICP-MS) using the ELAN 9000 ICP mass spectrometer (PerKinElmer, Canada). Samples were analyzed directly to measure dissolved Ag^+ ions and digested to measure total silver. Microwave digestion was performed as described by Wu *et al.* [48] and briefly summarized here. 10 mL concentrated nitric acid and 2 mL ultra-pure water were added to 1 g biofilm (wet weight) or 1 mL suspension and kept at room temperature overnight for pre-digestion. Microwave digestion was then carried out using ETHOS EZ Microwave Solvent Extraction Labstation (Milestone Inc., USA) with the following heating program: heat to 190 °C within 15 min and then hold at 190 °C for 10 min. The particle size and zeta potential of Ag-NPs was characterized using a Malvern Zetasizer Nano-ZS (Model: ZEN3600, Malvern Instruments Ltd, Worcestershire, UK). Since PVP dissolves in water completely, parameters of silver were adopted for the analysis: the refractive index was 2.0 and the absorption coefficient was 0.320 [49].

3. Results

3.1. Effects of Ag-NPs on reactor performance

No significant change in pollutant removal was observed after Ag-NP addition in any of the four reactors (Fig. 1A and B). COD removal rate was maintained at above 90% and ammonium removal was above 99% in each reactor all the time. Fig. 1C, D and E show the COD, ammonium and nitrate concentration change against time in four reactors on Day 63. Only slight difference in reaction kinetics has been observed. The majority of COD was removed from each reactor within the first 30 min and ammonium removal was completed within 4 h. The reaction rate constant for COD removal remained almost the same in all reactors (p-value > 0.05) after two months of Ag-NP addition. Nevertheless, minor difference can be seen in ammonium removal kinetics. The rate constant for ammonium removal decreased to 0.35 h^{-1} in the PVP control and 0.34 h^{-1} in the reactor with aged Ag-NP addition (p-value = 0.03), compared to the initial value of 0.55 h^{-1} before Ag-NP addition. However, this rate constant increased to 0.66 h^{-1} in the reactor fed with fresh Ag-NPs (p-value = 0.11). The rate constant decreased slightly to 0.48 h^{-1} in reactor fed with Ag^+ ion released from fresh Ag-NPs (p-value = 0.18). Fresh Ag-NP helped to maintain the ammonium oxidizing reaction rate in the reactors. However, it should be noted that if the biomass concentration is taken into account, the ammonium uptake rate decreased in all reactors, compared with the initial values (p-value < 0.05). The initial ammonium update rate was 3.0 mg N g^{-1} VSS $^{-1}$; the ammonium uptake rate decreased to 2.2, 2.0, 1.3, 2.3 mg N g^{-1} VSS h^{-1} in reactor fed with PVP, Aged Ag-NPs, fresh Ag-NPs and Ag^+ ion, respectively. Similarly, the reactor fed with fresh Ag-NPs also had the lowest COD uptake rate. This indicates that the metabolic activity of microbes in the reactor fed with fresh Ag-NPs didn't increase proportionally to the biomass concentration. As a matter of fact, the ammonium oxidizing reaction rate constant in the reactor fed with fresh Ag-NPs stayed the highest (above 0.6 h^{-1}) among all the reactors during the two-month treatment period. The ammonium uptake rate in the reactor fed with fresh Ag-NPs was also the highest (2.5 mg N g^{-1} VSS h^{-1}) in all four reactors on Day 22, before the significant biomass increase occurred. It appeared that nutrient uptake rates in the reactor fed with fresh Ag-NPs hit a bottleneck when the biomass and silver accumulation increased significantly.

3.2. Ag-NPs improved sludge settleability and increased biomass concentration

The biomass concentration in the reactor with fresh Ag-NPs increased significantly after two months of operation as indicated by mixed liquor suspended solids (MLSS) concentration in Fig. 2A. MLSS concentration reached the peak of 4866 mg/L on Day 56 in the reactor with fresh Ag-NPs while stabilized at about 2400 mg/L in the others. Meanwhile, the sludge volume index (SVI) in this reactor is much lower than the others as well (Fig. 2B). SVI stabilized at about 100 mL/g in the reactor with fresh Ag-NPs and stayed below 220 mL/g in reactors fed with aged Ag-NPs and Ag^+ released from fresh Ag-NPs. However, SVI increased to 295.6 mL/g in PVP control and remained above 240 mL/g until the end of the test. Big chucks of sludge can be seen by naked eye in the PVP control (irregular shape with the longest side over 2 cm). Sludge flocs may be physically bridged by of the PVP polymer and therefore they form chucks that are very light and fluffy, which makes them difficult to settle down. This is not hard to explain since PVP can help Ag-NPs to disperse in water using the same mechanism. Sludge flocs are bigger in the reactor with fresh Ag-NPs. All the reactors have one peak with chord length below 5 μm, while the reactor with fresh Ag-NPs has another major peak at about 20 μm (Fig. 2C). The density of sludge flocs in this reactor is also slightly larger than those in the other reactors (Fig. S1). More extracellular polymeric substances (EPS) were produced by the PVP control, especially protein (Fig. 2D). This also contributes to the poor settleability of the sludge in the PVP control, since excess EPS will result in sludge bulking [50].

3.3. Ag-NPs helped to maintain compositional diversity of the microbial community

Bacteria families vary in each sample as shown in Fig. 3A. After the thirty-day startup stage (sample S1), the microbial community composition changed slightly from the initial inoculum (sample S0). More significant shifts in community structure can be seen in the samples at the end of the test (S4-S7). Based on the principal coordinate analysis (PCoA) with weighted unifrac (Fig. 3B), along the axis accounts for 72% of variance samples S0 and S1 are close to each other while samples S4-S7 are clustered together. This divergence from the initial inoculum is very likely caused by operation time period in the lab since laboratory condition is different from that in the full-scale wastewater treatment plant, which is confirmed by the canonical correspondence analysis (CCA) as shown in Fig. 3C. If looking closer into axes PC2 and PC3 (Fig. 3C), it can be seen that sample S6 (reactor with fresh Ag-NPs, day 64) locates the closest to the initial inoculum S0. S6 is also close to sample S1, which is the initial state before Ag-NP addition for all reactors.

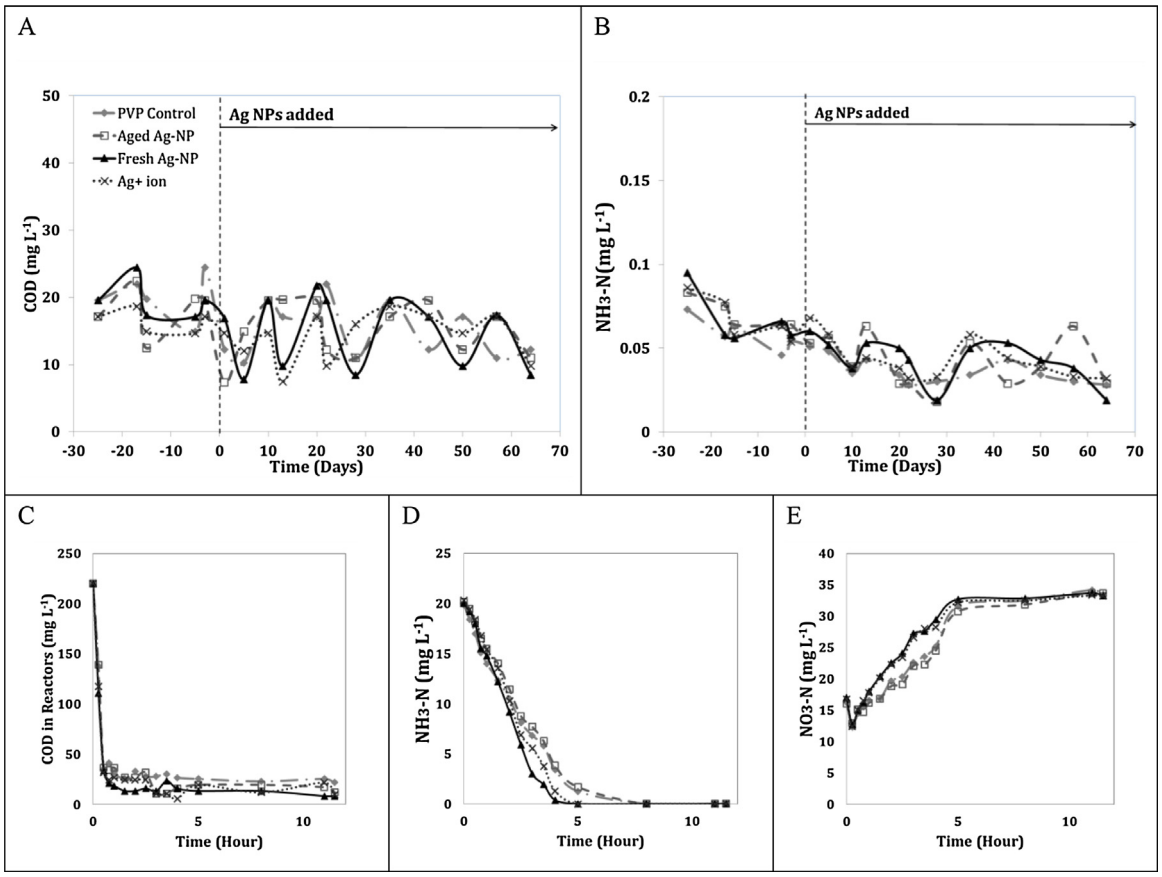

Fig. 1. Performance of each reactor. (A) Efluent COD concentration; (B) Effluent ammonium concentration; (C) COD removal kinetics; (D) Ammonium removal kinetics; (E) Nitrate production kinetics.

This indicates that fresh Ag-NP may help to maintain the microbial community.

CCA triplot in Fig. 3C illustrated the significant correlations ($p = 0.021$) between dominant bacteria families (accounting for over 70% sequence abundance in each sample) and the test conditions: operation time, silver accumulation and silver added as the nanoparticle form (NP added). Correlation of dominant bacteria families with silver added as Ag^+ ion was less significant and no significant correlation with PVP addition was seen in CCA. The 'Time' vector almost overlap with the F1 axes and the 'NP added' vector almost overlap with the F2 axes. Based on the CCA silver accumulation was positively associated with time and NP added, while time and NP added were almost orthogonal. The two axes F1 and F2 combined explained 92% of the variances in microbial community, indicating that time, silver added as the NP form and silver accumulation are the major factors shaping the microbial community. The family *Xanthomonadaceae* (F14) located close to the origin of the plot, indicating that this family has little response to the test conditions. Dominant families *Comamonadaceae* and *Rhodocyclaceae* (F9 and F10) fell near the F1 axes, indicating that time is the major factor that resulted in the changes of these two families. F2 (a family in the order of *Cytophagales*) and F8 (*Sphingomonadaceae*) increased with silver accumulation. *Cytophagales* has been reported to dominant in certain conditions with high metal concentration and may potentially be used in phytoremediation [51]. *Sphingomonadaceae* is a family of bacteria with sphingolipids in the outer membrane. Sphingolipids may potentially work as signaling molecules, although the endogenous functions of sphingolipids in bacteria are still unknown [52]. This signaling pathway may likely be associated with the synergetic effects of maintaining community diversity found in this study.

Table 1
Diversity index of microbial community and functional genes.

Sample	Microbial community (bacteria families)		Functional genes	
	Richness	Evenness	Richness	Evenness
S0	126	0.554	–	–
S1	122	0.407	904	0.796
S2	104	0.381	813	0.802
S3	93	0.296	906	0.797
S4	88	0.538	888	0.796
S5	86	0.558	891	0.796
S6	97	0.626	918	0.796
S7	110	0.552	915	0.795

In terms of diversity indices (richness and evenness, Table 1), both richness and evenness were lost at the beginning of the Ag-NP addition (S2, reactor with aged Ag-NPs, day 8; S3, reactor with fresh Ag-NPs, day 14). However, the loss of evenness recovered with time. After two months of Ag-NP addition, fresh Ag-NP (S6) maintained the highest evenness without relatively less loss in richness compared with the initial inoculum (S0).

3.4. Ag-NPs helped to maintain functional diversity of the microbial community

In terms of functional diversity, reactor with fresh Ag-NPs (S6) has the highest number of genes detected (Fig. 3D) and richness (Table 1). Evenness is almost identical for all samples for functional

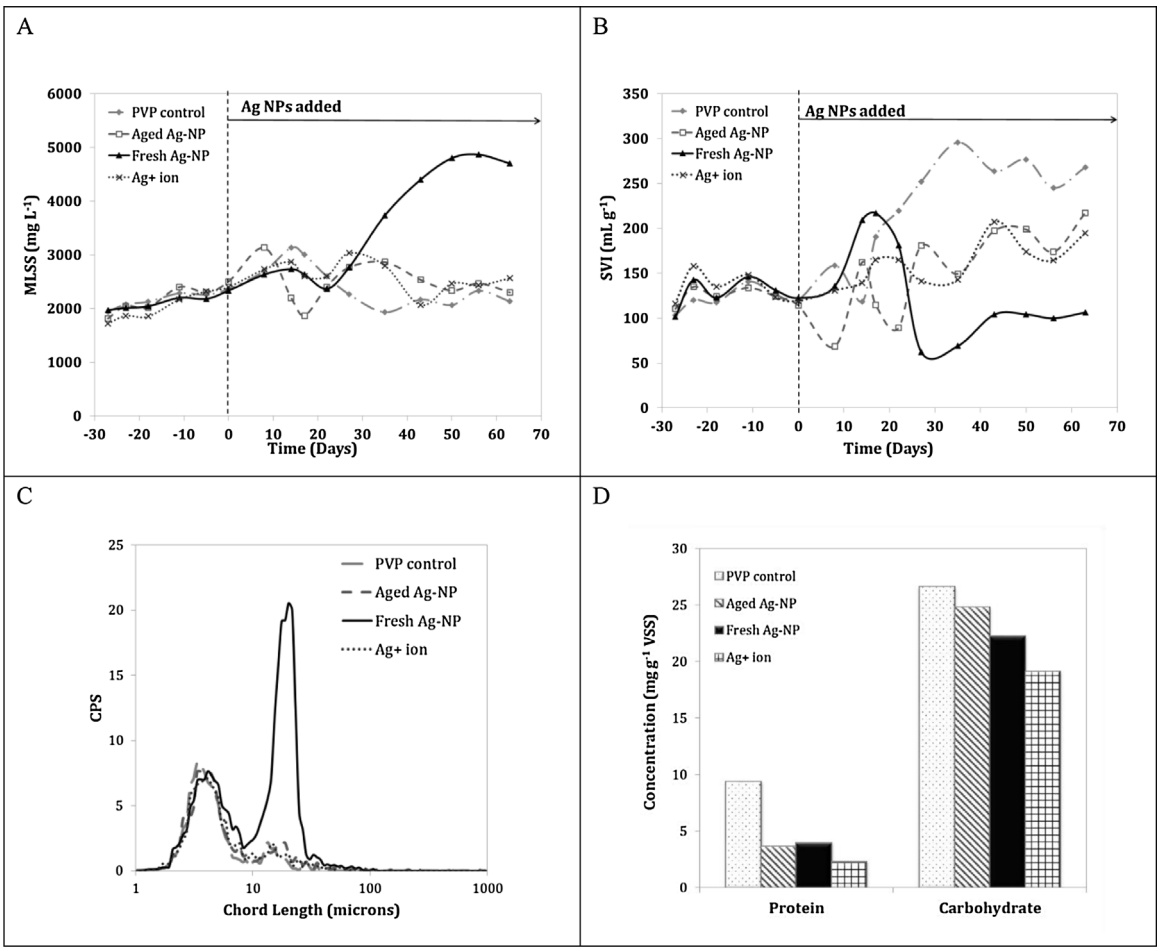

Fig. 2. Sludge property in each reactor. (A) MLSS concentration; (B) SVI; (C) Floc size on day 64; (D) Sludge EPS concentration on day 64.

Table 2
Particle characterization and silver dissolution.

	Particle size (nm)	Zeta potential in synthetic wastewater (mV)	Silver ion concentration in influent (mg/L)
Aged Ag-NP	74.2 ± 5.1	−9.37 ± 1.50	0.70 ± 0.10
Fresh Ag-NP	67.9 ± 1.0	−4.17 ± 1.03	0.10 ± 0.02

genes, which is consistent with the fact that there is no significant change in gene distribution in each function category. Detrended correspondence analysis (DCA) showed that (Fig. 3E) samples are clustered by time period along DCA1 and samples with Ag-NP addition (S5-7) are closer to the initial state (S1) compared to the PVP control (S4). This indicates that Ag-NP addition helped to maintain the functional diversity of the microbial community in the reactors. Fresh Ag-NP even increased functional diversity in terms of richness. Heatmap of the top 30 abundant functional genes also indicated that S6 (reactor with fresh Ag-NPs, day 64) and S7 (reactor with Ag^+ released from fresh Ag-NPs, day 64) are clustered with the initial state S1 (Fig. 3F). Some antioxidant enzyme and metal and stress resistant genes has been elevated by fresh Ag-NPs (S6). More significant increase in these genes was seen in sample S5 (reactor with aged Ag-NPs, day 64), which makes sense since aged Ag-NPs release more Ag^+ ions and Ag^+ ion plays an important role in the toxicity of Ag-NPs [3,53–55]. Particle size and zeta potential of aged and fresh Ag-NPs are at the same scale as shown in Table 2. The data also confirmed that much more Ag^+ ion was released from Aged Ag-NPs.

3.5. Silver species accumulation and release

Silver concentration in effluent and sludge was monitored. Dissolved Ag^+ ion concentration in the effluent in all reactors with Ag-NP addition remained below 0.01 mg/L all the time (Fig. 4A). Total silver release with effluent was the highest (0.017 mg/L) on the first day of Ag-NP addition in the reactor with Ag^+ ion released from fresh Ag-NPs. The reactor adapted to the silver addition within two weeks and total silver release never exceeded 0.01 mg/L thereafter. Total silver release increased with time in the reactors fed with fresh and aged Ag-NP and reached 0.76 and 0.89 mg/L respectively at the end of the test. The only difference is the release increased in steps in the reactor with fresh Ag-NPs, indicating that this reactor has more capacity to maintain Ag-NPs in the sludge, which is consistent with the total silver accumulation in sludge (Fig. 4B). Silver accumulation in sludge appeared to be periodical to some extent which is coupled with biomass concentration change. When the accumulation reached a threshold and the cells started to die, more silver is released through effluent and after the concentration in sludge decreased after the release, cell growth recovered and a new round of accumulation starts. More biomass

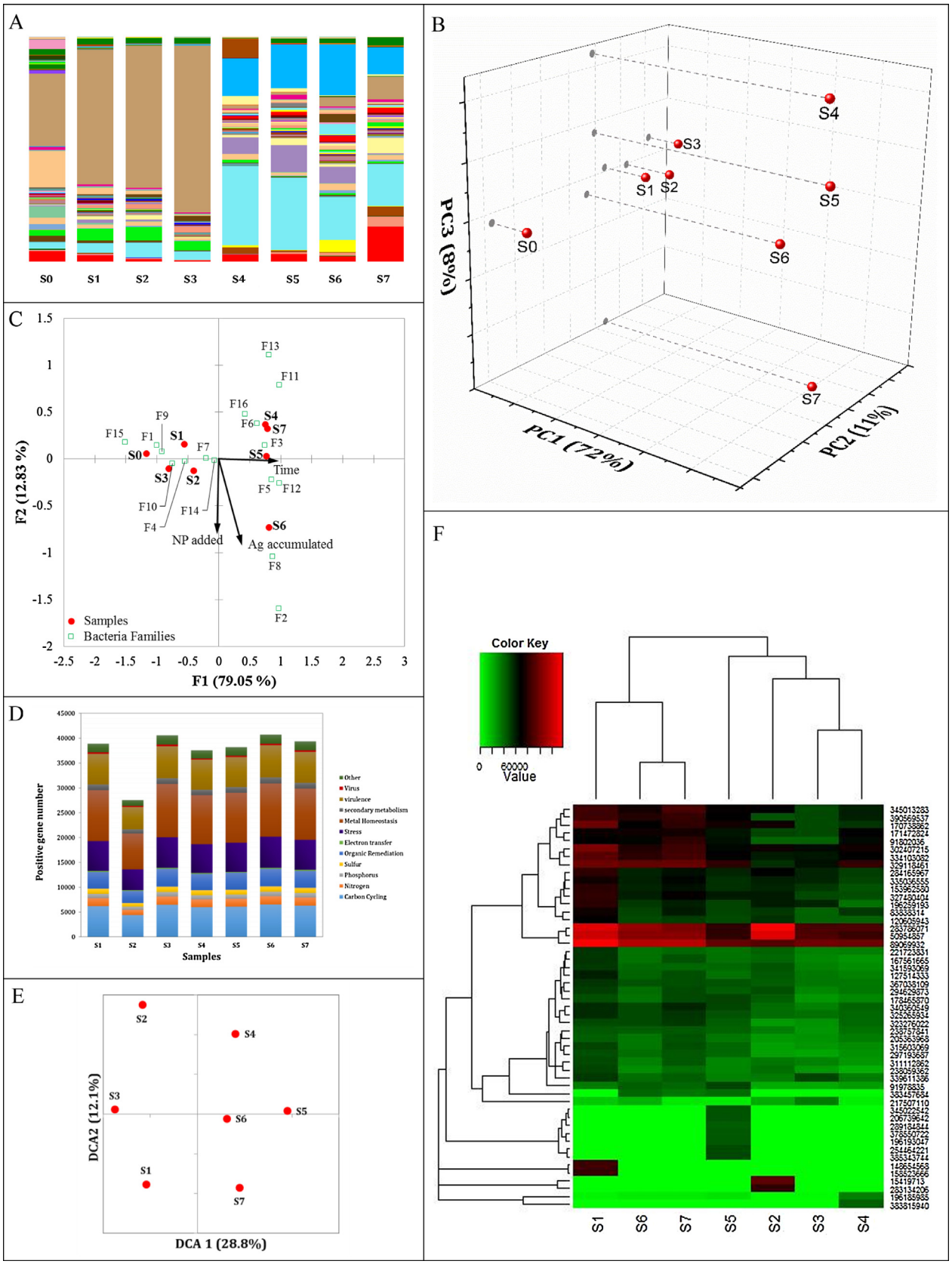

Fig. 3. Microbial diversity analysis. (A) Relative abundance of bacteria families; (B) principal coordinate analysis (PCoA) of microbial community diversity; (C) Canonical correspondence analysis (CCA) triplot of dominant bacteria families and test conditions; (D) The number of genes detected in each functional category; (E) Detrended correspondence analysis of functional gene diversity; (F) Heatmap of the top 30 abundant functional genes; Sample S0: The initial activated sludge inoculum; S1: Sludge after the thirty-day startup stage; S2: Sludge in reactor with aged Ag-NPs, day 8; S3: Sludge in reactor with fresh Ag-NPs, day 14; S4: Sludge in reactor with only PVP as control, day 64; S5: Sludge in reactor with aged Ag-NPs, day 64; S6: Sludge in reactor with fresh Ag-NPs, day 64; S7: Sludge in reactor with Ag+ released from fresh Ag-NPs, day 64.

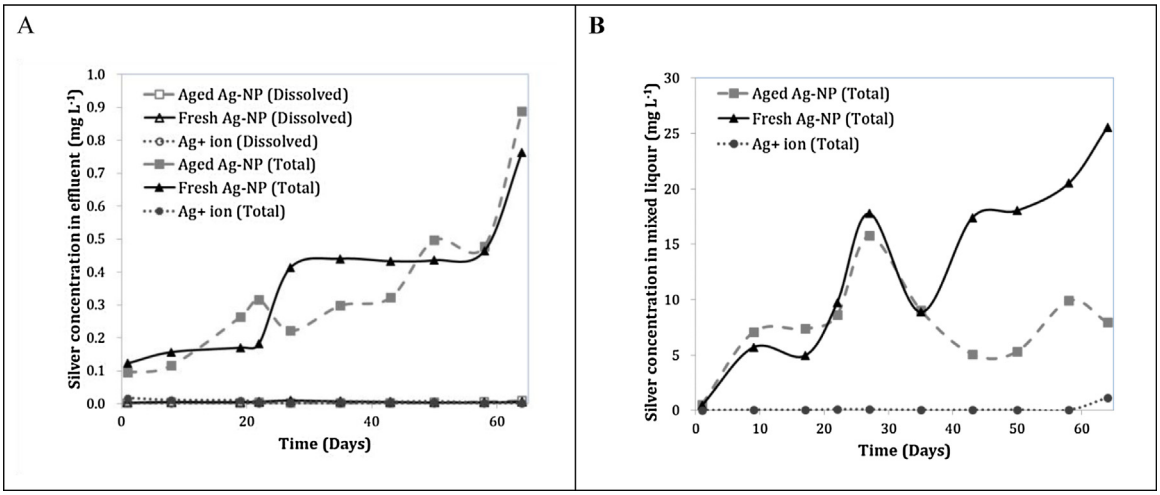

Fig. 4. Silver species accumulation and release. (A) Silver realease via effluent; (B) Silver accumulation in activated sludge. Silver concentrations in effluent and sludge from the PVP control reactor were both below the detection limit.

was maintained in the reactor with fresh Ag-NPs and more silver was accumulated in the sludge in that reactor.

4. Discussion

There is ample evidence that bacteria growth can be stimulated when sublethal concentration of antimicrobial agents are applied [56,57]. An inverted U-shaped dose-response relationship, so called hormesis model, have been reported for many antibiotics [58]. Most of these research were done with pure-cultured bacteria. Similar dose response has also been seen in studies with Ag-NPs [6–9]. However, controversy exists and these findings did not attract a fair amount of attention. Majority of literature reports non-significant to adverse effects of Ag-NP when tested at the mg/L scale [3,18,59–61]. In this study, increase in biomass concentration was clearly observed in reactors with fresh Ag-NPs and it was stably repeated in several batch of tests under similar Ag-NP concentrations. Results in this study and literature indicate that the effect of Ag-NP may conform to the hormesis model as well. When tested with pure-cultured bacteria, the hormetic effects were less significant and difficult to replicate. However, when tested with sophisticated bacteria community such as activated sludge used in this study, the positive effects appeared to be evident and stable. The maintenance of community diversity played a very important role in this hormetic effect observed here. The increased microbial community diversity by fresh Ag-NP is the most significant phenomenon observed besides the increase in biomass concentration. Acute effects at the beginning of addition come from loss of both richness and evenness. This is also consistent with the hormesis model where initial decrease in growth was followed by the adaptive rebound response [58]. More experiments with systematically designed dosages and treatment time need to be carried out to confirm the hormesis model in Ag-NP toxicology.

In this study, better maintenance of sludge in the reactor may play a more important role other than stimulation of growth. In the reactor with fresh Ag-NPs, increased floc size and density led to better settleability and therefore less sludge was lost through effluent withdraw and more biomass was accumulated in the reactor. In addition, higher diversity of the microbial community makes the microbes more resistant to stress [62,63]. As a result, the increased survival contributes to the higher biomass concentration. It has been reported that nano zero-valent iron can be used to control sludge bulking [64], and Ag-NPs can be another option. This is also supported by the fact that nutrient uptake rates didn't increase with biomass concentration in the reactor fed with fresh Ag-NPs. More cells were maintained in the reactor but not all of them are metabolically active or they may not be in their most active state. However, it should be noted that the lack of sufficient nutrients in the reactor might also contribute to the bottleneck in nutrient uptake rate increase.

Without considering time, the microbial community structure in samples with fresh and aged Ag-NPs closer resembled the initial inoculum, compared to the sample with only Ag^+ ion released from fresh Ag-NPs. This indicated that the nanoparticle form may play a more important role in the maintenance of microbial community diversity. In addition, Ag^+ ion doesn't improve sludge settling and increase biomass concentration in the reactor. However, in terms of reactor performance, especially ammonium removal kinetics, the reactor with Ag^+ ion performed more similar to the reactor with fresh Ag-NPs instead of aged Ag-NPs. This indicates, to some extent, that the released Ag^+ ion played an important role in maintaining the pollutant removal capacity. To better understand the mechanism behind the positive effects of Ag-NPs, tests with equal Ag^+ ion concentration but different nanoparticle concentration can be run in the future, along with additional control without treatment to verify the effects of operation time period. Longer startup stage can also be considered. High influent ammonium concentration may help to test if Ag-NP can improve high-ammonium concentration removal.

Hormtic effects can be triggered by many kinds of toxic substances, such as radiation and chemical reagents including antibiotics. The term "hormesis" was initially used to describe effects caused by low dose radiation and is now generally used to describe the inverted U-shape biological dose-response to stress [56,65]. Reports on the hormetic dose-response to antibiotics date back to the 1950s and various antibiotics were found to be able to cause hormetic effects [56,57]. Low-intensity pulsed ultrasound (LIPUS) was also found to stimulate cell growth and antibody production, although high dose of ultrasound can kill cells [66]. Agents that can cause hormetic effects can be classified into two types: 1) chemical toxins that can leave residuals and accumulate in cells, such as Ag-NP and other antibiotics; 2) agents that can physically change cells and leave no residuals, such as LIPUS. Radiation is more complicated and can cause both physical and chemical effects. Ag-NPs are chemically toxic to cells and can be accumulated in or near cells, therefore, the positive effects caused by Ag-NPs can be limited. This is consistent with the result that biomass concentration started to decrease in the reactor fed with fresh Ag-NPs

at the end of the test, which may result from the high concentration of silver species accumulated in the sludge. Longer term of operation will help to verify the effects of silver accumulation. This is also consistent with the result that nutrient uptake didn't increase with biomass concentration in the reactor fed with fresh Ag-NPs. The toxicity of Ag-NPs may induce a dormancy state of the cells therefore no enhanced metabolic activity was observed although more cells were maintained in the reactor. LIPUS work with a different mechanism as it increase cell permeability which lead to better circulation, faster cell metabolism and enhanced antibody secretion. Because no residual is left by LIPUS, repeated stimulation with LIPUS can cause more significant beneficial effects than Ag-NP. Substances that can cause hormetic effects can also be classified as non-selective and selective. LIPUS can work on many kinds of cells and is non-selective. Ag-NP, which can be taken as a broad-spectrum antibiotics, is also relatively non-selective. Therefore, agents such as Ag-NP and LIPUS tend to work equally on various kinds of cells. This explains why Ag-NP increased the diversity of microbial community in activated sludge without significant changes in the distribution of genes in each functional group. On the contrary, selective agents such as narrow-spectrum antibiotics, are more inclined to cause selective effects such as changes in dominant species in microbial community. As reported in literature, changes in gene expression caused by subinhibitory concentration of broad-spectrum antibiotics are often termed with "enhance", "increase" or "stimulation"; effects caused by subinhibitory concentration of narrow-spectrum antibiotics are often described by the word "inhibited" or "reduced" [56]. In regard to a complicated ecosystem such as activated sludge in wastewater treatment, it is easier for non-selective agents with no residuals (such as LIPUS) to cause significant beneficial effects; non-selective agents with residuals (such as Ag-NP) ranks the second, but there are limitations caused by the toxicity and accumulation; it is relatively difficult for selective agents with residuals (such as narrow-spectrum antibiotics) to cause significant beneficial effects on such a complicated microbial community.

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