Supplementary Information

Long-Term Performances of Enhanced Biological Phosphorus Removal with Increasing Concentrations of Silver Nanoparticles and Ions

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Synthetic Wastewater. The synthetic wastewater included stock SOP solution named "P - water", "concentration solution", "trace-element solution" (adapted from Smolders et al.[1]), a certain amount of carbon source and tap water. The P-water contained (g/L): 23.5 KH_2PO_4 and 17.6 $K_2HPO_4 \cdot 3H_2O$. The concentrated solution contained (g/L): 25.88 peptone, 4.24 yeast extract, 33.94 $MgCl_2 \cdot 6H_2O$, 19.09 $MgSO_4 \cdot 7H_2O$, 8.91 $CaCl_2 \cdot 2H_2O$, 8.91 NH_4Cl and 0.11 allylthiourea (nitrification inhibitor). The trace-element solution contained (g/L): 1.50 $FeCl_3 \cdot 6H_2O$, 0.03 $CuSO_4 \cdot 5H_2O$, 0.12 $MnCl_2 \cdot 4H_2O$, 0.06 $Na_2MoO_4 \cdot 2H_2O$, 0.12 $ZnSO_4 \cdot 7H_2O$, 0.15 $CoCl_2 \cdot 6H_2O$, 0.18 KI, 0.15 H_3BO_3 and 10 ethylenediamine tetraacetic acid. One liter of synthetic wastewater contained 1.94 mL of P-water, 2.14 mL of concentrated solution, 0.27 mL of trace-element solution, and 0.267 mL of acetic acid. The synthetic wastewater final composition of the influent contained (mg/L): 55.38 peptone, 9.07 yeast extract, 72.63 $MgCl_2 \cdot 6H_2O$, 40.85 $MgSO_4 \cdot 7H_2O$, 19.07 $CaCl_2 \cdot 2H_2O$, 19.07 NH_4Cl, 0.24 allylthiourea, 0.41 $FeCl_3 \cdot 6H_2O$, 0.01 $CuSO_4 \cdot 5H_2O$, 0.03 $MnCl_2 \cdot 4H_2O$, 0.02 $Na_2MoO_4 \cdot 2H_2O$, 0.03 $ZnSO_4 \cdot 7H_2O$, 0.04 $CoCl_2 \cdot 6H_2O$, 0.05 KI, 0.04 H_3BO_3, 2.70 ethylenediamine tetraacetic acid, 45.59 KH_2PO_4, 34.14 $K_2HPO_4 \cdot 3H_2O$ and 280 acetic acid.

Operation of Parent Sequencing Batch Reactors (SBRs). Activated sludge used in this study was cultured with synthetic wastewater in a series of anaerobic-aerobic SBRs, which had a working volume of 4 L each and operated with a cycle of 8 h consisting of 2 h anaerobic and 3h aerobic periods, followed by 1h settling, 10 min decanting and 110 min idle periods. All reactors were covered with aluminium foil, maintained at 21±1 ^{o}C and constantly mixed with a magnetic stirrer except the settling, decanting and idle periods. The influent pH in each reactor was adjusted to 7.5 by adding 4M NaOH or 4M HCl. In the aerobic stage, air was provided by an aerator using an on/off control system, and the dissolved oxygen (DO) concentration was maintained at around 6 mg/L. After the settling period, 3 L of the supernatant was discharged, and replaced with 3 L of fresh synthetic wastewater during the next initial 10 min of the anaerobic time. The mixed liquid suspended solid (MLSS) was controlled at 3200 ± 190 mg/L in the parent SBRs. Sludge was wasted at the end of aerobic periods to keep the solids retention time (SRT) at approximately 10 d. The initial chemical oxygen demand (COD) concentration in each SBR was increased progressively over a 30 d period

from around 80 to approximately 300 mg/L. After around 120 d of acclimatization, the phosphorus anaerobic release and aerobic uptake in each SBR reached relatively stable.

Determination of Activities of Exopolyphosphatase (PPX) and Polyphosphate Kinase (PPK). The determination of PPX activity was conducted according to the references.[2, 3] The reaction was carried out at 30 ^{o}C after adding 50 uL crude extracts to the reaction mixture containing 0.5 M Tris-HCl buffer (pH 7.4), 5 mM $MgCl_2$ and 2.5 mM p-nitrophenyl phosphate. After 45 min incubation, 2 mL of 0.5 M KOH was added to terminate the reaction, followed by measuring the absorbance at 405 nm. The specific PPX activity was defined as the production of umol p-nitrophenol/(min•mg protein).

The assay of polyphosphate (poly-P) utilization was used to determine the PPK activity.[4] The reaction, in a final volume of 1 mL, contained 100 mM Tris-HCl (pH 7.4), 8 mM MgCl2, 200 mM D-glucose, 0.5 mM NADP, 150 ug of Sigma Type 45 poly-P, 1 unit of HK, 1 unit of G6P-DH, and 150 uL of crude extracts. Ap5A was included in the assay to inhibit adenylate kinase. The reaction was started by adding the ADP resulting in a final concentration of 1 mM. The produced NADPH was measured spectrophotometrically at 340 nm.[5] The specific PPK activity was determined as the production of umol NADPH/(min•mg protein).

Analysis of Bacterial Community Structure. PCR-DGGE was first utilized to analyze the bacterial community structure in the EBPR systems. Bacterial genomic DNA of activated sludge was extracted using the FastDNA Kit (BIO 101; Vista, CA, USA) according to the manufacturer's instructions. The 16S rDNA variable V3 region of the extracted DNA was amplified with primers 338f with a GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGACTCCTACGGGAGGCAGCAG-3') and 518r (5'-ATTACCGCGGCTGCTGG-3').[6] The detailed procedures of PCR and DGGE were according to the literature.[7] All prominent bands were excised from gel sequenced via an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, USA) and deposited into the GenBank database under accession numbers JQ954834-JQ954839. Then, the closest matching sequences were searched using the BLAST program. The

quantifications of polyphosphate accumulation organisms (PAO), glycogen accumulation organisms (GAO) and total bacteria in activated sludge were conducted by fluorescence in situ hybridization (FISH) analysis.

Microbial Quantitative Changes Analyzed by FISH with 16S rRNA-targeted Oligonucleotide Probes. The quantifications of PAO, GAO and total bacteria in activated sludge were conducted by FISH analysis. Activated sludge obtained from the SBR-1, SBR-2 and SBR-3 was fixed with freshly prepared 4% paraformaldehyde for 8 h at 4 ^{o}C. After being rinsed with phosphate buffer (PBS, pH 7.2), 10 uL of samples were immobilized on gelatin coated glass slide, dehydrated in the ethanol serials (50%, 75%, 85% and 98%, 3 min per step), and finally dried in air. The following oligonucleotide probes, EUBMIX (containing EUB338, EUB338-II and EUB338-III, specific for most *Bacteria*), PAOMIX (containing PAO462, PAO651 and PAO846, specific for *Accumulibacter*) and GAOMIX (containing GAOQ431, GAOQ989 and GB_G2, TFO_DF218, TFO_DF618, specific for *Candidatus Competibacter phosphatis*), were used for hybridization and listed in Table S1 (Supplementary Information). These probes were commercially synthesized and labeled with FITC, AMCA, Cy3 at the 5' end, respectively. Hybridization on the slide glass was performed according to the method of Amann et al. with slight modification.[8] Briefly, 20 uL of hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% sodium dodecyl sulfate, 35% deionized formamide and 0.2 ng probes) was hybridized with the fixed samples, and then the slides were incubated in a prewarmed Boekel InSlide Out Hybridization Oven (Boekel Scientific, USA) at 46 ^{o}C for 2 h, followed by a washing step at 48 ^{o}C for 20 min in a washing buffer (20 mM Tris-HCl (pH 7.2), 70 mM NaCl, 5 mM EDTA and 0.01% SDS). The washing buffer was removed by rinsing with sterile water and the slide was dried in air. FISH samples were finally observed using the Laser Scanning Confocal Microscope (Leica TCS SP2). Each sample had triplicated test, and five fields were chosen to take images for every test, which meant that there were 15 images for each sample used for FISH quantification. The abundance of Accumulibacter or *Candidatus Competibacter phosphatis* was determined as the mean image area targeted by PAOMIX or GAOMIX and that targeted by EUBMIX in the image analyzing software (Image-Pro Plus, V6.0, Media Cybernetics).

Measurement of AgNPs Dissolution in Synthetic Wastewater. The released Ag^+ in synthetic wastewater due to the dissolution of AgNPs was determined according to the literature with some modification.[9] Briefly, 5 vials containing the synthetic wastewater (pH 7.5) and AgNPs (0, 1, 3 and 5 mg/L) were shaken for 5 h at 21 ± 1 ^{o}C after 2h ultrasonication, and AgNPs were then removed by high speed centrifugation (12000 *g*) for 20 min. The supernatant was collected, filtered through 0.22 um mixed cellulose ester membrane, and determined by inductively coupled plasma optical emission spectrometry (ICP-OES, PerkinElmer Optima 2100 DV, USA) after acidified with 4% ultrahigh purity HNO_3.

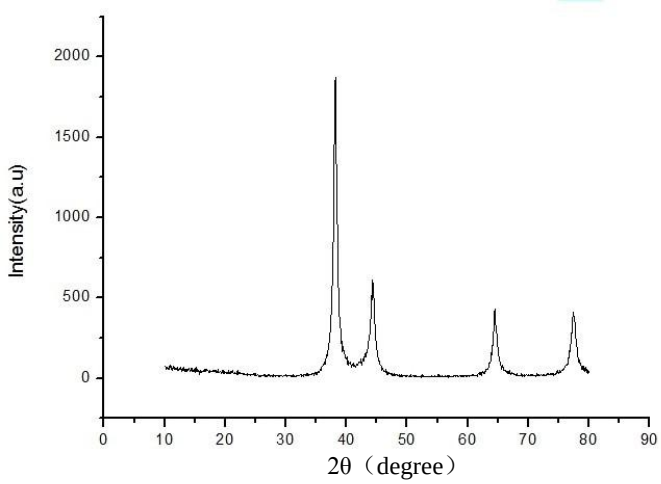

Fig. S1 X-ray diffraction (XRD) pattern of AgNPs used in this study.

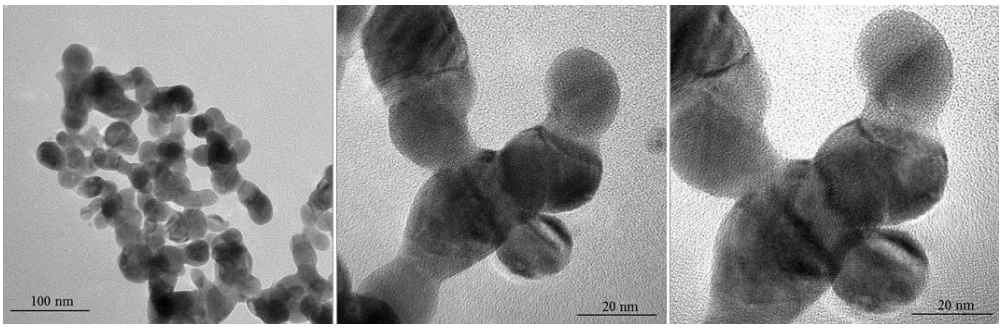

Fig. S2 Images of transmission electron microscope (TEM) of AgNPs used in this study.

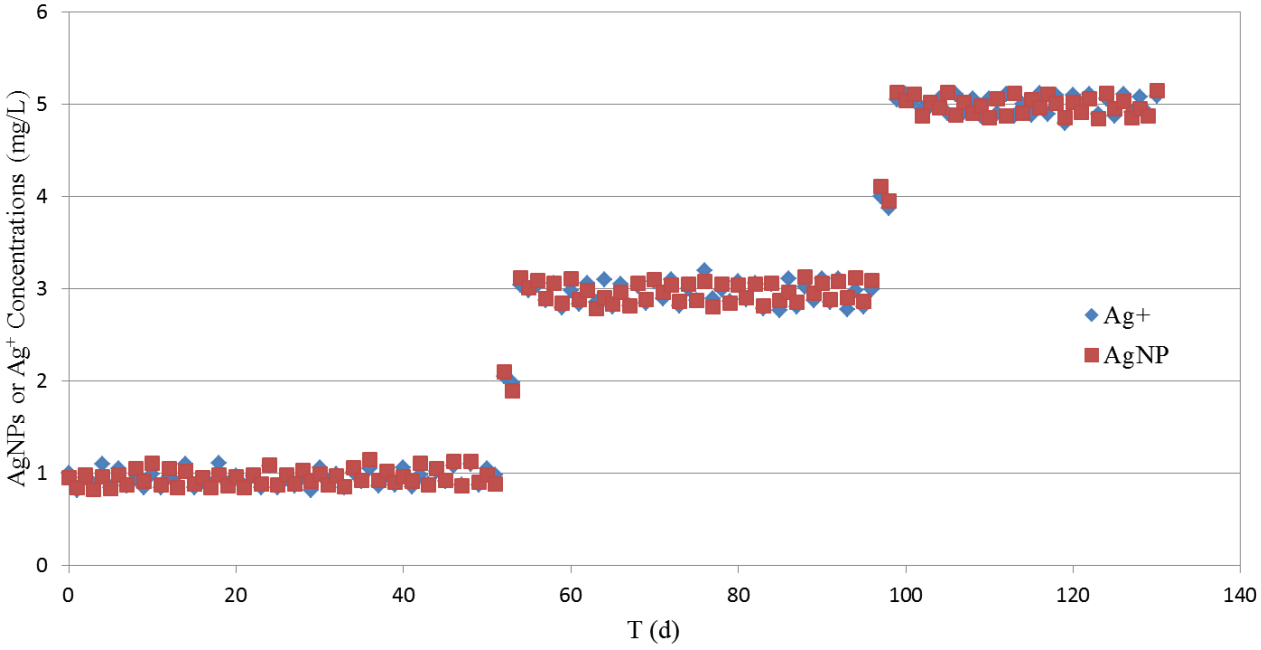

Fig. S3 The AgNPs or Ag^{+} concentration variation in the period of long-term exposure.

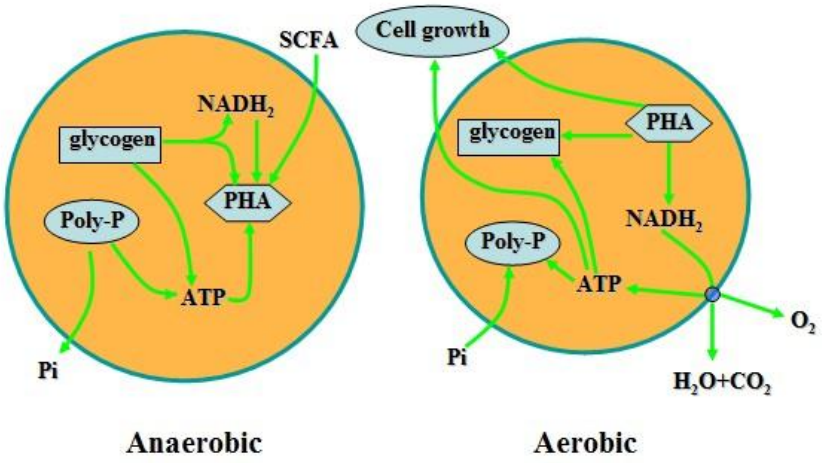

Fig. S4 The schematic diagram of main substrates metabolism involved in the anaerobic and aerobic stages of enhanced biological phosphorus removal.

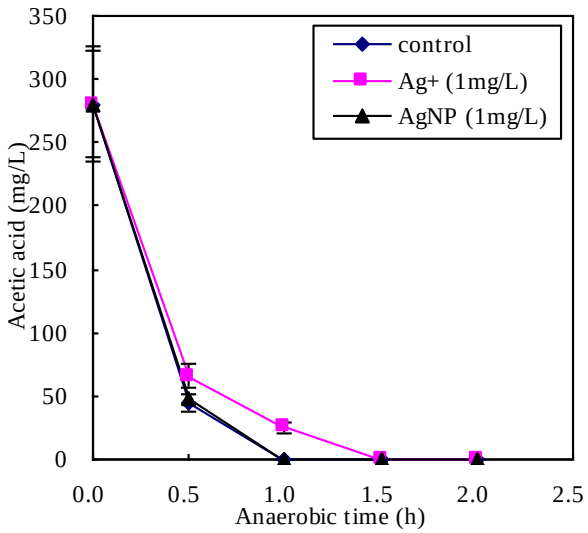

Fig. S5 The changes of acetic acid concentrations. Effects of 1 mg/L of Ag^{+} and AgNPs on the variations of acetic acid during the anaerobic time. Error bars represent standard deviations of triplicate measurements between day 1 - day 3.

Table S1. Oligonucleotide Probes Used in This Study

Probe	Specificity	Sequence (5'-3')	FA (%)
PAO462	*Rhodocyclus*-related PAO in *Betaproteobacteria*	CCGTCATCTACWCAGGGTATTAAC	20-35
PAO651	*Rhodocyclus*-related PAO in *Betaproteobacteria*	CCCTCTGCCAAACTCCAG	35
PAO846	*Rhodocyclus*-related PAO in *Betaproteobacteria*	GTTAGCTACGGCACTAAAAGG	35
GAO Q431	*Gammaproteobacteria Competibacter spp.*	TCCCCGCCTAAAGGGCTT	35
GAO Q989	*Gammaproteobacteria Competibacter spp.*	TTCCCCGGATGTCAAGGC	35
GB_G2	*Gammaproteobacteria Competibacter spp.*	TTCCCCAGATGTCAAGGC	35
TFO_DF218	*Defluvicoccus*-related TFO in *Alphaproteobacteria*	GAAGCCTTTGCCCCTCAG	20-35
TFO_DF618	*Defluvicoccus*-related TFO in *Alphaproteobacteria*	GCCTCACTTGTCTAACCG	20-35
DF988	*Defluvicoccus*-related DF in *Alphaproteobacteria*	GATACGACGCCCATGTCAAGGG	35
DF1020	*Defluvicoccus*-related DF in *Alphaproteobacteria*	CCGGCCGAACCGACTCCC	35
EUB338	Most Bacteria	GCTGCCTCCCGTAGGAGT	35
EUB338-Ⅱ	*Planctomycetales* and other Bacteria not detected by EUB338	GCAGCCACCCGTAGGTGT	35
EUB338-Ⅲ	*Verrucomicrobiales* and other Bacteria not detected by EUB338	GCTGCCACCCGTAGGTGT	35

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Table S2. The Statistical Analysis Results of the Effect of AgNPs and Ag^+ on Phosphorus Removal

Reactor	Concentration (mg/L)	Operational time	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
AgNPs	1	Day 1-3	0.27	7.71	0.63
Ag^+	1	Day 1-3	17.84	7.71	0.01
AgNPs	1	Day 4-50	1.51	4.41	0.24
Ag^+	1	Day 40-50	0.14	7.71	0.73
AgNPs	3	Day 51-95	0.92	4.41	0.35
Ag^+	3	Day 85-95	1.97	7.71	0.23
AgNPs	5	Day 96-130	2.34	4.60	0.15
Ag^+	5	Day 120-130	0.43	7.71	0.55

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Table S3. The Statistical Analysis Results of the Short-term (day 1-3) Effect of 1 mg/L AgNPs and Ag^+ on the Anaerobic and Aerobic Transformations of PHA between day 1 and day 3 (Compared with the Control)

	AgNPs			Ag^+		
	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
PHA anaerobic synthesis	3.32	7.71	0.14	6.90	7.71	0.06
PHA aerobic degradation	0.34	7.71	0.59	10.35	7.71	0.03

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Table S4. Effects of Ag^+ and AgNPs on the Activities of PPX and PPK [a]

Reactor	Concentration (mg/L)	Operational time	PPX[b]	PPK[c]
AgNPs	1	Day 1-3	0.08±0.02	0.31±0.03
Ag^+	1	Day 1-3	0.05±0.01	0.29±0.01
Control	0	Day 1-3	0.09±0.02	0.29±0.02
AgNPs	1	Day 40-50	0.09±0.01	0.31±0.04
Ag^+	1	Day 40-50	0.08±0.01	0.32±0.02
Control	0	Day 40-50	0.10±0.02	0.29±0.03
AgNPs	5	Day 120-130	0.08±0.01	0.30±0.03
Ag^+	5	Day 120-130	0.09±0.03	0.31±0.02
Control	0	Day 120-130	0.09±0.02	0.32±0.04

[a] The data reported are the averages and their standard deviations of triplicate tests.

[b] The unit is umol *p*-nitrophenol/(min·mg protein).

[c] The unit is umol NADPH/(min·mg protein).

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Table S5. DGGE Bands Based on the V3 Region of 16S rRNA Gene and their Closely Related Sequences

Band ID	Accession no.	Most closely related bacterial sequence		Identity
		Species and strain	Accession no.	(%)
1	JQ954834	Uncultured *Aeromonadaceae bacterium* clone 4.13h2	JN695859.1	99
2	JQ954835	Uncultured *Arcobacter sp.* clone OO.P2.OT.73.ab1	HQ821665.1	99
3	JQ954836	Uncultured *Candidatus Accumulibacter* sp. clone EMB clone_7	HM046420.1	98
4	JQ954837	Uncultured *beta proteobacterium* clone P-R68	JN038855.1	99
5	JQ954838	*Moraxella sp.* B62	JQ390117.1	100
6	JQ954839	*Pseudomonas sp.* qdcs28	JQ319068.1	100

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Table S6. The Statistical Analysis Results of the Effect of Ag^+ and AgNPs on EPS of Activated Sludge (Compared with the Control)

		AgNPs			Ag^+		
		$F_{observed}$	$F_{significance}$	$P_{(0.05)}$	$F_{observed}$	$F_{significance}$	$P_{(0.05)}$
Sudden addition (1 mg/L, 1-3 d)	Protein	0.29	7.71	0.62	0.08	7.71	0.79
	Carbonhydrate	4.03	7.71	0.12	0.82	7.71	0.42
	Total EPS	0.03	7.71	0.87	0.01	7.71	0.91
Long-term exposure (1 mg/L, 40-50 d)	Protein	0.08	7.71	0.80	61.33	7.71	1.40×10^{-3}
	Carbonhydrate	5.59	7.71	0.08	40.08	7.71	3.19×10^{-3}
	Total EPS	0.03	7.71	0.87	57.65	7.71	1.61×10^{-3}
Long-term exposure (5 mg/L, 120-130 d)	Protein	1.13	7.71	0.35	49.50	7.71	2.15×10^{-3}
	Carbonhydrate	5.15	7.71	0.09	48.52	7.71	2.23×10^{-3}
	Total EPS	0.32	7.71	0.60	48.75	7.71	2.21×10^{-3}

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