Effect of organic loading on phosphorus forms transformation and microbial community in continuous-flow A^2/O process

Yajing Li, Yaping Wu, Shaopo Wang and Liyuan Jia

ABSTRACT

A continuous-flow Anaerobic/Anoxic/Oxic (A^2/O) system was operated at different organic concentrations to systematically investigate the effect on the nutrient removal, secretion characteristics of extracellular polymer, phosphorus forms transformation and changes in functional flora in this system. The results showed that high organic loading was more conducive to promote the secretion of extracellular polymeric substance (EPS), the increase of polysaccharide content was more obvious compared with protein, the impact of organic loading on the components of loosely bound EPS (LB-EPS) was higher than that of tight-bound EPS (TB-EPS). Phosphorus in sludge floc mainly existed in the form of inorganic phosphorus (IP), and IP mainly existed in the form of apatite inorganic phosphorus (AP). High organic load showed higher phosphorus storage in EPS, and the phosphorus content in EPS was positively correlated with the content of EPS. Non-apatite phosphorus (NAIP) content played an important role in the extracellular dephosphorization. The abundance of *Nitrosomonas* and *Nitrospira* responsible for nitrification decreased with the increase in organic loading. The group of denitrifiers was large, and *Azospira* was the most abundant genus among them. *Dechloromonas*, *Acinetobacter*, *Povalibacter*, *Chryseolinea* and *Pirellula* were the functional genera closely associated with phosphorus removal.

Key words | continuous-flow A^2/O system, EPS, microbial community structure, organic loading, phosphorus forms distribution

Yajing Li (corresponding author)
Yaping Wu
Shaopo Wang
School of Environmental and Municipal Engineering,
TCU,
Tianjin 300384,
China
E-mail: *yajingli79@163.com*

Yajing Li
Shaopo Wang
Tianjin Key Laboratory of Aquatic Science and Technology,
Tianjin 300384,
China

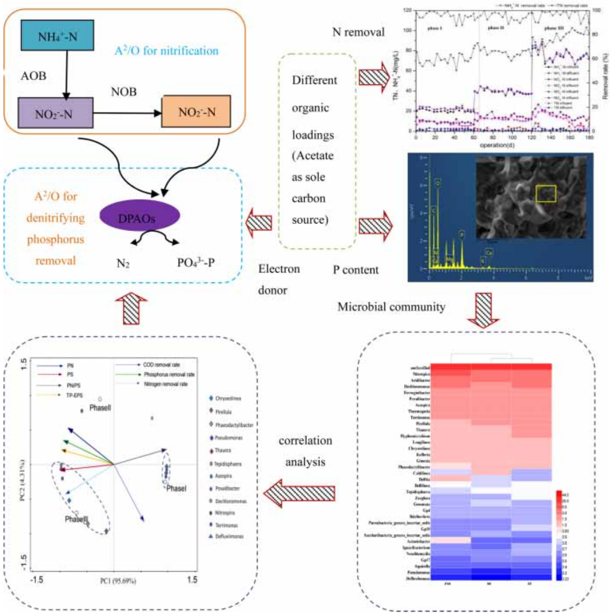
Liyuan Jia
Tianjin IKWEN Water Treatment Co., Ltd,
Tianjin 300000,
China

HIGHLIGHTS

- High organic loading promoted the secretion of EPS, the impact of organic loading on the components of LB-EPS was higher than that of TB-EPS.
- NAIP played an important role in the extracellular phosphorus removal.
- Influent organic loading shaped the bacterial community structure.
- The core functional genera in N and P removal were identified by correlation analysis.

doi: 10.2166/wst.2021.158

GRAPHICAL ABSTRACT



INTRODUCTION

Urban domestic sewage in China has the characteristics of large quantity and wide range of pollutant sources, leading to significant water quality fluctuation (Yu *et al.* 2014; Li *et al.* 2016; Zhang *et al.* 2017). The effect of organic loading on nitrogen and phosphorous removal in activated sludge system has been studied for many years (Szabo *et al.* 2017; Wang *et al.* 2018; Rusanowska *et al.* 2019; Wang *et al.* 2019; Gong *et al.* 2019; Zhang *et al.* 2020). The A/A/O process, or the A^2/O process, is the organic combination of biological nitrogen removal A_N/O process and biological phosphorus removal A_P/O process. The A^2/O process is a traditional biological nitrogen and phosphorus removal water treatment technology (Zhao *et al.* 2018), which has been widely used at home and abroad. The A^2/O process ranks the second and accounted for 15.30% among the 4,560 wastewater treatment plants by 2020 in China (Lan 2020). However most studies on phosphorus in the entire A^2/O synchronous biological nitrogen and phosphorus removal process remain at the level of total phosphorus, and there have been only limited research and discussion on the various forms of phosphorus and the migration and transformation rules among microbial cells, extracellular polymeric substance (EPS), and bulk liquid.

In recent years, studies have shown that EPS has an important effect on biological phosphorus removal and participate in the enhancement of biological phosphorus removal (Li *et al.* 2015; Long *et al.* 2017; Shi *et al.* 2017). The contribution of EPS to phosphorus uptake and the forms of accumulated extracellular phosphorus vary substantially in different studies, and the underlying mechanism of phosphorus transformation and transportation in EPS remains poorly understood (Geyik & Çeçen 2015; Yang *et al.* 2017; Zheng *et al.* 2019). Therefore, it is necessary to carry out an in-depth research on the influence of organic loading on continuous-flow A^2/O systems. Ways to remove various forms of phosphorus were determined by investigating the distribution and content changes of phosphorus in sludge flocs. Which forms of phosphorus are more easily absorbed and utilized by EPS, and which forms of phosphorus are more easily absorbed and utilized by cells, and the contribution rate of EPS and bacterial cells in the process of biological phosphorus removal were determined. The mechanism of biological phosphorus removal can be improved from the new exploration directions. Combined with the role of EPS in biological phosphorus removal, the adsorption of phosphorus by EPS can be maximized in practical engineering according to the wastewater properties and operation process to effectively and rapidly remove phosphorus.

In order to establish the internal relationship between the macroperformance and microstructure of the A^2/O

process, 16S rRNA sequencing technology will be used to compare and analyze the dominant flora structural evolution under different influent organic loading, and to discuss the role of the functional flora in the biological nitrogen and phosphorus removal system to explore the potential of further improving the effect of nitrogen and phosphorus removal, and aim to provide theoretical reference and technical support for the production and operation of wastewater treatment plants.

A continuous-flow A^2/O reactor was operated with different organic loadings for 6 months in this study. The objectives of this study were to: (1) contrast the C, N, P removal performances, and further explore the changes of total EPS and key component contents under different organic loadings, as well as the changes of sub-component characteristics of different EPS; (2) investigate the distribution and content of phosphorus forms inside and outside the cells in the sludge floc, analyze the migration and transformation rule of phosphorus with different forms, and understand the role of EPS in the phosphorus removal mechanism of A^2/O systems; (3) identify the key functional groups including the microbes responsible for the removal of nitrogen, phosphorus. Based on the results, an attempt was made to seek a mechanism interpretation between organic loading and microbehavior of the A^2/O process.

MATERIALS AND METHODS

Setup of continuous-flow reactor

The experimental device is shown in Figure 1. It was composed of anaerobic tank, anoxic tank, aerobic tank and sedimentation tank, all of which were made of Plexiglass. The anaerobic tank was a cylinder with an inner diameter of 23.5 cm, a height of 34.0 cm and an effective volume of 12.8 L. The anoxic tank was a cylinder with an inner diameter of 29.5 cm, a height of 34.0 cm and an effective volume of 19.8 L. The aerobic pool size was: length (L) × width (B) × height (H) = 38.0 cm × 38.0 cm × 45.0 cm, and the effective volume was 65.0 L. An aerator was provided at the bottom of aerobic pool. After the aerobic tank, a vertical sedimentation tank was set up, and a water outlet weir was set above the sedimentation tank.

Synthetic wastewater and reactor operation

The activated sludge used was taken from the A^2/O process of a wastewater treatment plant (WWTP) in Tianjin. The component of the synthetic wastewater used in this study is shown in Table 1. Moreover a 1.0 mL/L trace elements solution was added into the synthetic wastewater, the composition of which was the same as that described by Li (2019).

The experimental period included three phases with the different organic concentrations, which were named phase I, phase II and phase III, respectively. Acetate was used as the sole carbon source, the water inflow was 188.0 L/d, the hydraulic retention times of anaerobic tank, anoxic tank and aerobic tank were 1.6 h, 2.5 h and 8.3 h respectively, the total hydraulic retention time of the A^2/O system was 12.4 h, the return sludge ratio was 100%, and the nitrate recycling ratio was 250%. Sludge retention time (SRT) was controlled at 15d and sludge concentration was about 3,000 mg/L. DO was controlled at about 3.0 mg/L by aeration.

EPS extraction protocol

EPS extraction was carried out using an ultrasonic-cation exchange resin method (Li *et al.* 2020). (1) 50 mL sludge samples were centrifuged at 3,000 r/min for 20 min, and

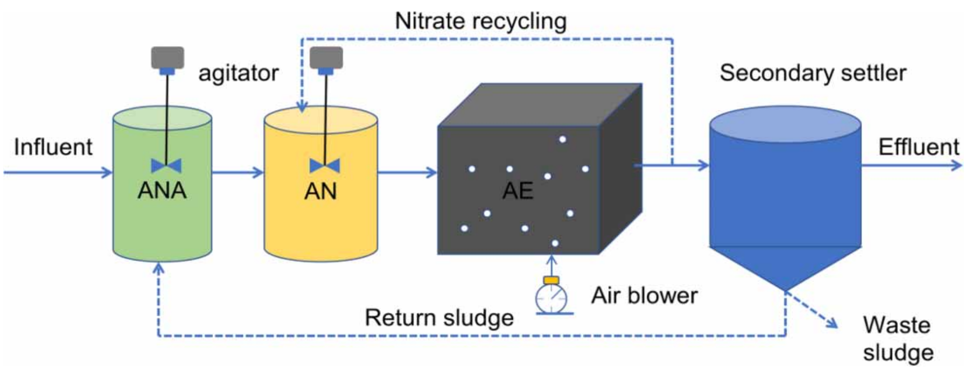


Figure 1 | Schematic experimental device of the A^2/O system.

Table 1 | Operation parameters and wastewater characteristics

Parameter/Component	Phase I (Low organic load)	Phase II (Medium organic load)	Phase III (High organic load)
Operating day	1–60	61–120	121–180
CH_3COONa (mg/L)	128.2	256.4	512.8
NH_4Cl (mg/L)	76.4	152.8	305.6
KH_2PO_4 (mg/L)	17.6	35.1	70.2
$MgSO_4$ (mg/L)	38.0	76.0	154.0
$CaCl_2$ (mg/L)	14.0	28.0	56.0
$NaHCO_3$ (mg/L)	50.0	100.0	200.0
Trace elements solution (mL/L)	1.0	1.0	1.0
COD (mg/L)	100	200	400
NH_4^+-N (mg N/L)	20	40	80
PO_4^{3-}-P (mg P/L)	4	8	15

the supernatant was used as LB-EPS. (2) The precipitated sludge after centrifugation was remixed with PBS solution for ultrasound (21 kHz, 40 W, 2 min). (3) The dose of CER (Dower Marathon C, Na^+ form, 20–50 mesh, Fluka 91,973) and the mixing speed of magnetic stirrer were adjusted to 60 g/g·VSS and 1,000 rpm, respectively, with a 4 h extraction time. (4) The mixture was centrifuged at 12,500 r/min for 20 min, and the supernatant produced was TB-EPS. Polysaccharide (PS) was determined by anthrone colorimetry, while protein (PN) was determined using a Coomassie bright blue colorimetry (Zhou *et al.* 2013; Tan *et al.* 2014). DNA nucleic acid content was determined by ultraviolet absorption method (Sheng *et al.* 2010).

Microscopy

The morphologies of sludge before and after EPS extraction were examined using a high-resolution scanning electron microscope (SEM) (model JEM 7800F, Japan). Sludge samples were fixed with 2.5% glutaraldehyde in 0.1 M PBS. Subsequently, the samples were washed and dehydrated in a series of ethanol solutions (50, 70, 80, 90, and 100%). Dewatered samples were dried by the critical point method. The dried sludge samples were further sputter coated with gold for SEM observation.

Method for determination of morphological phosphorus

The contents of total phosphorus (TP), organic phosphorus (OP), inorganic phosphorus (IP), non-apatite inorganic phosphorus (NAIP) and apatite inorganic phosphorus (AP) in sludge and bacterial cells were measured using a European standard SMT protocol. The contents of different forms of phosphorus in EPS were calculated by difference subtraction method. The specific extraction method was referred from Guo *et al.* (2015).

High-throughput sequencing

To analyze the microbial characteristic, sludge samples were collected from the reactor during the different operation phases and stored at −20 °C after removing the supernatant until DNA extraction. Microbial DNA extraction was carried out based on the protocols of Shanghai Sangon Biotech Co., Ltd. 16S rRNA high-throughput sequencing was carried out using the Illumina MiSeq system (Illumina MiSeq, USA). The V3–V4 region of 16S rRNA gene was selected by polymerase chain reactor (PCR) with a forward primer 341F: CCTACGGGNGGCWGCAG and a reverse primer of 805R: GACTACHVGGGTATCTAATCC (Li *et al.* 2018). PCR amplification, the extraction and purification of amplicons, and sequencing were performed on an Illumina MiSeq platform at Shanghai Sangon Biotech Co., Ltd.

Statistical analysis

The correlations between environmental variables and the relative abundances of different genera were determined by Pearson correlation using IBM SPSS Statistics 25. Principal component analysis (PCA) was carried out via the Canoco 5.0 software to assess the correlation between environmental parameters and community abundance at the genus level.

RESULTS AND DISCUSSION

C, N and P removal performance under different organic loadings

Figure 2(a) shows the COD removal efficiency in different operating phases. The effluent COD concentration all met the national standard A of level I of urban sewage treatment plant pollutant discharge standard (GB18918-2002), which indicated that the A^2/O process has a good impact resistance capacity.

As shown in Figure 2(b), phosphorus removal efficiency gradually increased from 63.2 to 77.6% with the increase of organic loading. However, the ratio of phosphorus uptake in anoxic zone decreased gradually from 56.6 to 42.1%. The

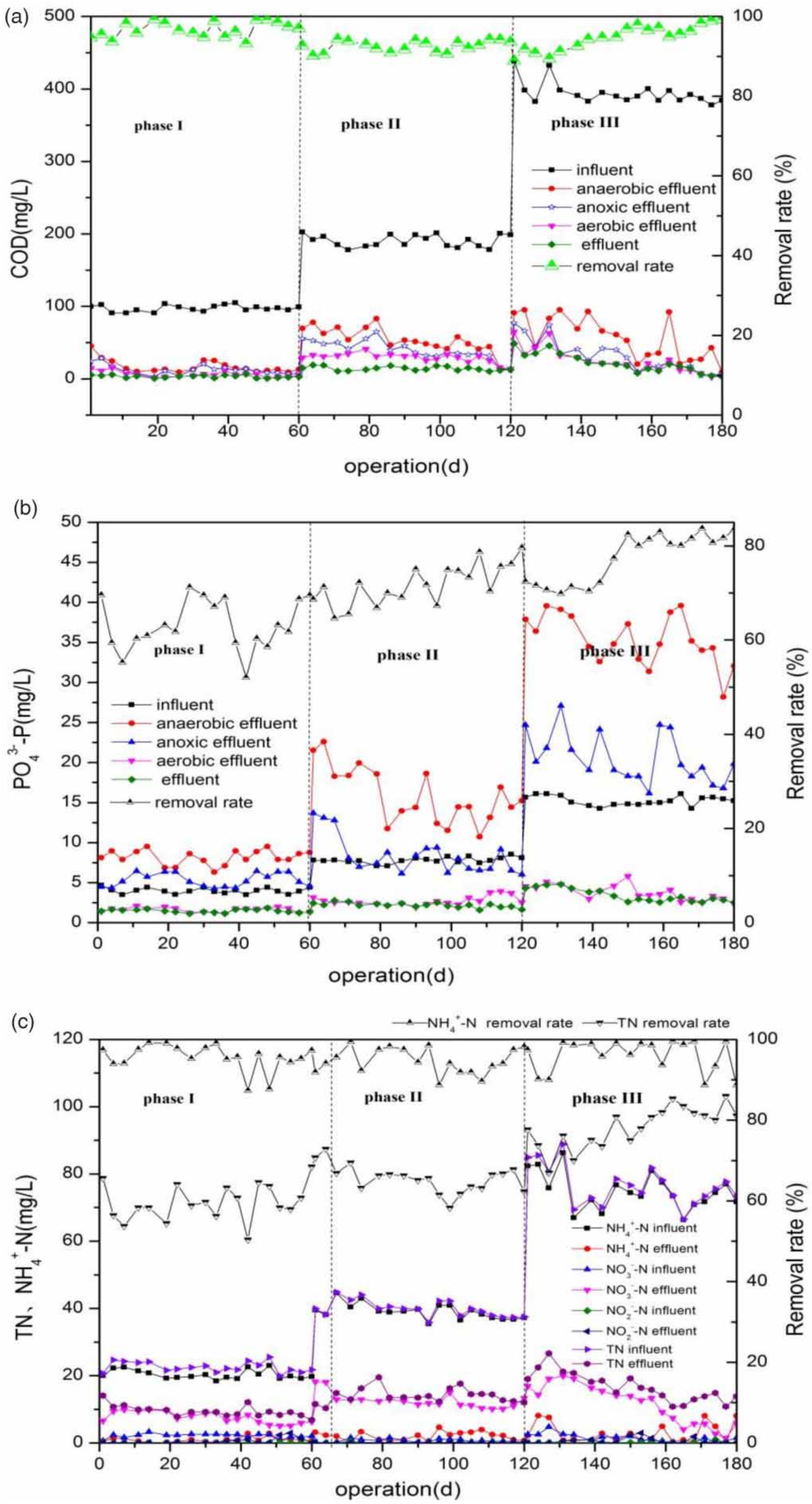


Figure 2 | Profiles of C, N and P removal characteristics of the A^2/O system over the 180 days' operational period ((a) COD; (b) PO_4^{3-}-P; (c) TN NH_4^+-N and NO_X^--N).

reason was that higher COD concentration entering the anoxic zone would reduce the phosphorus uptake capacity of the system.

It can be seen from Figure 2(c), different influent organic loadings had little impact on the nitrification performance of the A^2/O system. This was directly related to the sufficient hydraulic retention time and dissolved oxygen concentration in the aerobic zone of the system (Rong *et al.* 2019). NO_3^--N was higher under low and medium organic loading, which resulted in the reduction of TN removal rate. No significant accumulation of nitrite nitrogen was found in three phases.

Secreted characteristics of extracellular polymers

As shown in Table 2, the total EPS concentration showed an increasing trend with the increase in organic loading. TB-EPS accounted for (59.2 ± 7.1) %, (65.7 ± 5.6) % and (70.4 ± 2.4) % of the total EPS. Therefore, TB-EPS was an important component of EPS. Among them, PN/PS in LB-EPS varied from 7.72 to 0.57, and PN/PS in TB-EPS varied from 1.57 to 0.58, indicating that the impact of organic loading on the components of LB-EPS was higher than that of TB-EPS. Meanwhile, PS and PN were positively correlated with organic loading, and the change in trend of PS was more obvious compared with PN. This was consistent with the findings of Geyik & Çeçen (2016). This was because the carbon source was more sufficient under the conditions of high organic loading, and the bacteria could not make full use of it in time, so the surplus carbon source was converted into polysaccharide EPS. In summary, it can be seen that organic loading had an obvious effect on the components and contents of EPS.

The distribution and content of different phosphorus forms in sludge

As can be seen from Table 3, TP content per unit mass of sludge floc increased with the increase in influent organic loading, which was basically 19.36–43.81 mg/g. Among them, OP content was 4.47–15.70 mg/g, IP content was 13.52–31.65 mg/g, NAIP content was 2.64–11.63 mg/g, apatite inorganic phosphorus (AP) content was 10.99–20.08 mg/g. The mass concentration ratio of IP to TP was 69.83–72.24%, and the ratio of OP to TP was 23.08–35.86%, which indicated that phosphorus in sludge was mainly in the form of IP. The mass concentration ratio of NAIP to IP was 19.53–37.68%, and the ratio of AP to IP was 62.58–82.40%, which indicated that IP in sludge mainly existed in the form of AP.

Under different influent organic loading, TP in EPS accounted for 18.96–38.59% of that in sludge floc, OP and IP in EPS respectively accounted for 12.74–19.69% and 15.96–21.54% of that in sludge floc, which indicated that OP and IP mainly existed in cells. With the increase in influent organic loading, there was a higher phosphorus content in EPS, and TP content increased from 2.06–3.94 to 10.02–17.21 mg/g, increased by 4.37–4.86 times. It may be that more EPS had stronger phosphorus adsorption/binding performance. Phosphorus atomic percentage in EPS were studied using SEM and energy dispersive spectrometry (EDS), as shown in Figure 3. The results showed that it

Table 2 | Comparison of extracellular polymer secretion under different organic loadings

	$LB/mg{\cdot}g^{-1}$ VSS			$TB/mg{\cdot}g^{-1}$ VSS			
Sample	**Polysaccharides**	**Proteins**	**PN/PS**	**Polysaccharides**	**Proteins**	**PN/PS**	**Nucleic acids/$\mu g{\cdot}g^{-1}$ VSS**
L-A1	2.66 ± 0.18	2.71 ± 0.12	0.98	3.45 ± 0.17	7.23 ± 0.30	2.10	2.64 ± 0.20
L-A2	1.28 ± 0.08	3.36 ± 0.18	2.63	4.31 ± 0.21	6.72 ± 0.40	1.56	4.15 ± 0.43
L-O3	1.56 ± 0.12	12.05 ± 1.02	7.72	7.69 ± 0.47	12.05 ± 1.09	1.57	3.99 ± 0.27
M-A1	14.81 ± 1.42	29.99 ± 1.23	2.02	39.32 ± 2.42	33.19 ± 1.71	0.84	13.11 ± 1.22
M-A2	15.25 ± 1.22	17.70 ± 1.27	1.16	35.12 ± 1.22	27.87 ± 1.32	0.79	13.60 ± 0.80
M-O3	15.29 ± 1.80	24.09 ± 1.32	1.58	34.94 ± 2.27	27.39 ± 1.09	0.78	12.35 ± 1.32
H-A1	38.72 ± 4.42	9.27 ± 1.81	0.24	41.08 ± 4.34	23.08 ± 4.42	0.56	24.25 ± 2.01
H-A2	34.87 ± 5.81	14.28 ± 2.09	0.41	42.84 ± 6.94	28.55 ± 8.20	0.67	10.00 ± 1.02
H-O3	35.19 ± 4.72	20.2 ± 2.27	0.57	43.21 ± 8.20	25.08 ± 5.24	0.58	12.60 ± 1.20

Note: L-A1, L-A2, L-O3 were the samples taken from anaerobic, anoxic, aerobic tank in phase I.
M-A1, M-A2, M-O3 were the samples taken from anaerobic, anoxic, aerobic tank in phase II.
H-A1, H-A2, H-O3 were the samples taken from anaerobic, anoxic, aerobic tank in phase III.

Table 3 | Changes of phosphorus forms in sludge under different organic loadings

Sample	TP	OP	IP	NAIP	AP
L-A1	20.80 ± 1.71	5.09 ± 0.12	14.19 ± 0.98	3.13 ± 0.17	11.28 ± 1.30
L-A2	19.36 ± 1.32	4.47 ± 0.18	13.52 ± 2.63	2.64 ± 0.21	11.14 ± 1.40
L-O3	20.78 ± 1.09	5.70 ± 1.02	13.96 ± 2.72	2.98 ± 0.47	10.99 ± 1.09
L-A1′	2.06 ± 0.12	0.52 ± 0.23	1.25 ± 0.22	2.67 ± 0.42	0.53 ± 0.03
L-A2′	4.02 ± 1.22	0.64 ± 0.27	3.09 ± 0.76	2.22 ± 0.22	0.20 ± 0.02
L-O3′	3.94 ± 1.80	0.57 ± 0.32	3.40 ± 0.58	2.54 ± 0.27	0.80 ± 0.17
M-A1	26.09 ± 0.61	9.21 ± 0.15	17.46 ± 0.31	5.49 ± 0.01	11.67 ± 0.40
M-A2	25.12 ± 0.51	9.49 ± 0.20	15.71 ± 0.32	4.21 ± 0.01	11.40 ± 0.34
M-O3	24.71 ± 0.49	8.94 ± 0.17	15.76 ± 0.13	3.92 ± 0.01	11.95 ± 0.31
M-A1′	6.94 ± 0.02	1.20 ± 0.01	5.00 ± 0.05	4.23 ± 0.06	0.77 ± 0.01
M-A2′	7.63 ± 0.03	3.20 ± 0.01	3.97 ± 0.02	2.87 ± 0.03	1.21 ± 0.01
M-O3′	9.23 ± 0.01	4.34 ± 0.02	3.93 ± 0.02	2.42 ± 0.03	1.21 ± 0.02
H-A1	43.81 ± 0.86	10.25 ± 0.12	30.76 ± 0.37	11.59 ± 0.09	19.25 ± 0.20
H-A2	43.17 ± 0.44	13.10 ± 0.19	31.44 ± 0.29	11.32 ± 0.12	20.08 ± 0.17
H-O3	44.60 ± 0.72	15.70 ± 0.20	31.65 ± 0.24	11.64 ± 0.07	19.93 ± 0.23
H-A1′	10.02 ± 0.17	2.32 ± 0.03	7.94 ± 0.01	6.10 ± 0.16	1.32 ± 0.01
H-A2′	15.70 ± 0.29	6.59 ± 0.06	9.47 ± 0.07	5.76 ± 0.11	3.57 ± 0.02
H-O3′	17.21 ± 0.12	8.78 ± 0.08	9.06 ± 0.12	5.72 ± 0.09	4.02 ± 0.02

Note: L-A1, L-A2, L-O3 were the sludge samples taken from anaerobic, anoxic, aerobic tank in phase I.
M-A1, M-A2, M-O3 were the sludge samples taken from anaerobic, anoxic, aerobic tank in phase II.
H-A1, H-A2, H-O3 were the sludge samples taken from anaerobic, anoxic, aerobic tank in phase III.
L-A1′, L-A2′, L-O3′ were the EPS samples taken from anaerobic, anoxic, aerobic tank in phase I.
M-A1′, M-A2′, M-O3′ were the EPS samples taken from anaerobic, anoxic, aerobic tank in phase II.
H-A1′, H-A2′, H-O3′ were the EPS samples taken from anaerobic, anoxic, aerobic tank in phase III.

increased from 2.24 to 5.57% and 9.05% with the increase in organic loading and the extracellular phosphorus content accounted for 18.96%, 25.95% and 38.28% of the total sludge phosphorus content by calculation, which was largely consistent with the data in Table 3.

Moreover, the content of OP increased from 0.52–0.64 to 2.32–8.78 mg/g, the content of IP increased from 1.25–3.40 to 7.94–9.47 mg/g, the content of NAIP increased from 2.22–2.54 to 5.72–6.10 mg/g and the content of AP increased from 0.53–0.80 to 1.32–4.02 mg/g. The above results showed that the IP content of EPS was higher than that of OP under different influent loads, and the IP content of EPS mainly existed in NAIP form. With the increase in influent organic loads, there were significant differences in the changes of phosphorus content in different forms in EPS. Excluding the mechanism of phosphate precipitation, whether the formation of phosphorus in the EPS was affected by the microbial population structure and metabolic mode was to be determined and is described in the next section.

Analysis of dominant bacteria and functional bacteria

Dominant bacteria at the genus levels in different phases

In order to reveal the structural changes of the microbial community under different organic loadings, the relative abundance of dominant genera (>1% abundance in at least one sample) was analyzed, the total relative abundances of these genera in anaerobic, anoxic, and aerobic chambers were 75.83, 75.98, and 75.25% in phase I, 80.14, 81.27, and 81.17% in phase II, 72.69, 73.19, and 70.39% in phase III respectively.

As shown in Figure 4, the species of microbes and their relative abundance clearly showed high similarity among sludge samples from different chambers of A^2/O system at the same loading stage. The dominant bacteria in phase I were *Nitrospira* (5.34–6.62%), *Aridibacter* (2.98–3.49%), *Dechloromonas* (2.78–3.01%), *Ferruginibacter* (1.95–2.25%), *Povalibacter* (1.97–2.12%) and *Azospira* (1.92–2.12%). The

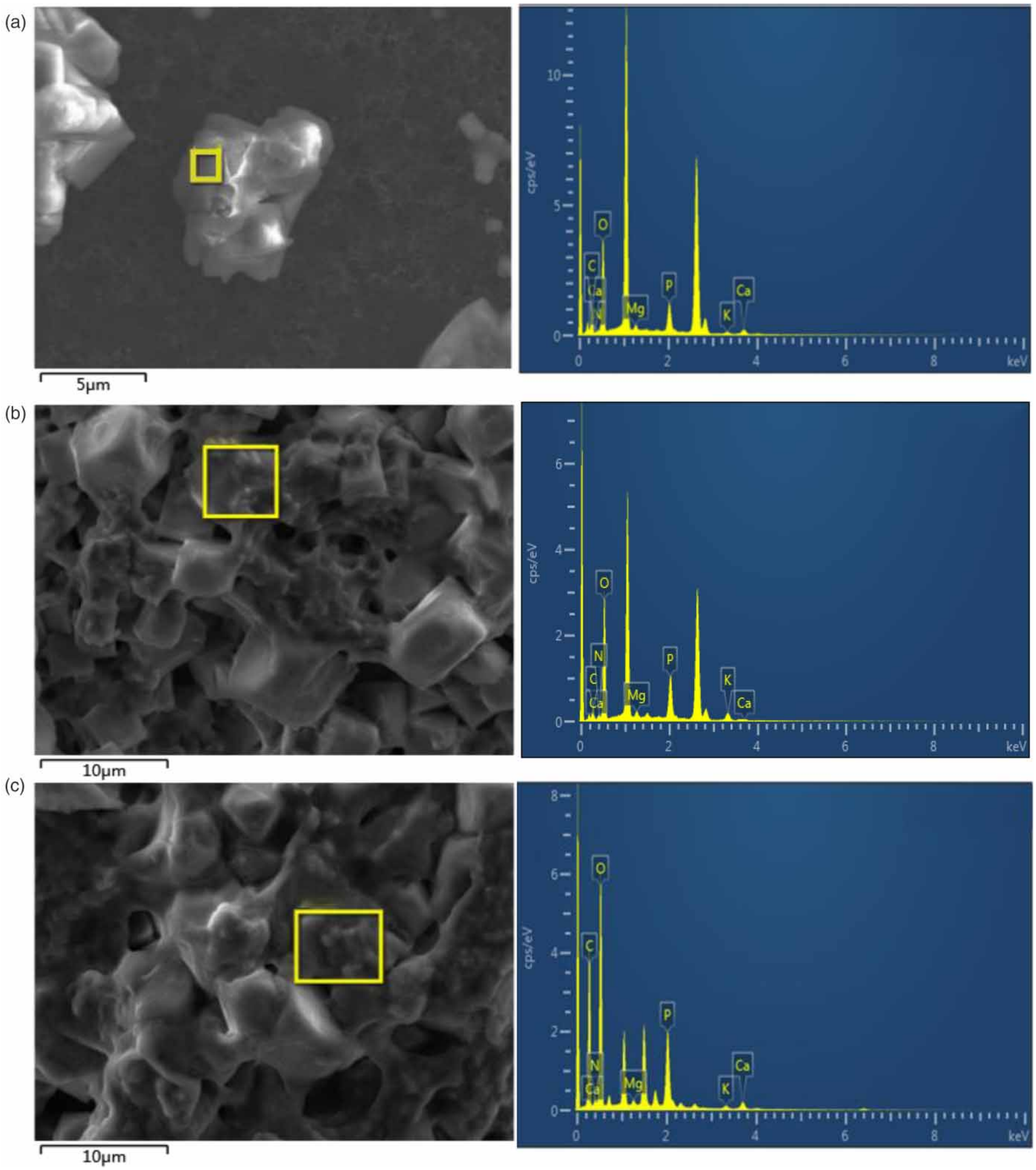


Figure 3 | EDS analysis of sludge samples with different organic loading. (a) Sludge samples in phase I. (b) Sludge samples in phase II. (c) Sludge samples in phase III.

dominant bacteria in phase II were *Azospira* (19.96–23.81%), *Nitrospira* (3.18–3.86%), *Povalibacter* (2.9–4.01%) and *Dechloromonas* (2.81–3.02%). The dominant bacteria in phase III were *Azospira* (9.77–10.83%), *Kofleria* (2.30–2.64%), *Chryseolinea* (2.22–2.58%), *Romboutsia* (2.17–2.65%) and *Nitrospira* (2.17–2.65%). With the increase in organic loading, Nitrospira's relative abundance decreased, Azospira's relative abundance increased firstly and then decreased, which differed from findings of a previous study (Guo *et al.* 2017) due to different influent and operating conditions. Dechloromonas's relative abundance did not change too much. At the same time, *Kofleria*, *Chryseolinea* and

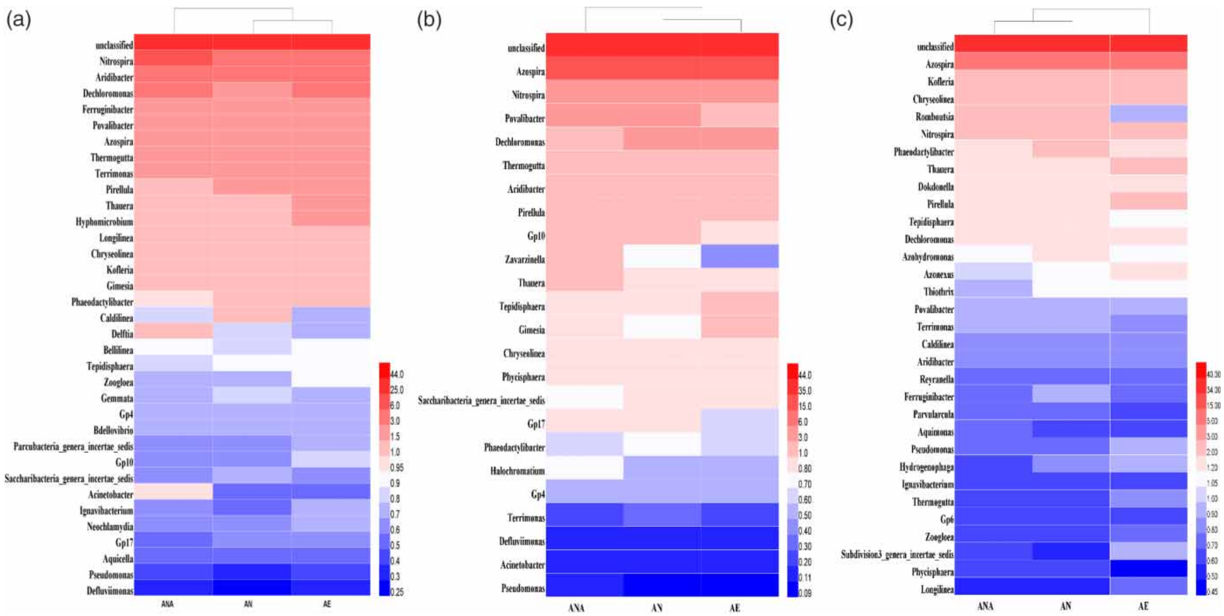


Figure 4 | Heat map of the dominant genera in each sample. The color intensity (percentage) in each panel represents the percentage of a genus in a sample, referring to the color key at the right. (a) Samples in phase I. (b) Samples in phase II. (c) Samples in phase III.

Romboutsia became the dominant bacteria instead of *Aridibacter* and *Ferruginibacter*, which showed a system-depended trait.

Functional bacteria at the genus levels in different phases

Nitrification, denitrification and phosphorus removal caused by the metabolism of functional bacteria were discussed for our A^2/O bioreactors.

Nitrification. According to the results of sludge samples under different organic loadings, only *Nitrosomonas* was detected as AOB and *Nitrospira* was detected as NOB. It showed that the nitrifying bacteria community structure was unitary and the diversity was low in this system, which may be that feed water and operating conditions of the system were selective for microorganisms, it was not conducive to the growth of other nitrifying bacteria. As shown in Table 4, *Nitrosomonas* and *Nitrospira* accounted for 0.02–0.24% and 2.17–6.62% respectively, the abundance decreased with the increase in organic loading. *Nitrospira* got the highest relative abundance (6.62%) in phase I, which was not conducive to short-cut nitrification to save the carbon source (Massara *et al.* 2017). This resulted in an accumulation of nitrate nitrogen corresponding to the poor removal performance of nitrate.

Denitrification. The group of denitrifiers was large and versatile, with at least 20 different taxa in our reactor. Such as *Azospira*, *Thauera*, *Tepidisphaera*, *Phaeodactylibacter*, *Dokdonella*, *Thiothrix*, *Terrimonas*, *Ferruginibacter*, *Pseudomonas*, *Zoogloea* and *Acinetobacter*. The cumulative relative read abundance of denitrifiers was about 20–25% in the three phases. Most of these genera are mixotrophic bacteria. Specific relative abundance in three phases is shown in Table 4. The results showed that the removal of nitrogen in the system was mainly accomplished by the traditional aerobic nitrification and anoxic denitrification, thus ensuring the removal efficiency of TN. Especially, *Azospira* was the most abundant genus, and accounted for 2.12, 22.05 and 9.77% with the increasing of organic loads. This was closely related to the strong denitrification performance of the system.

Phosphorus removal. By sorting the data of activated sludge 16S rRNA at each phase, it was found that the known Rhodocyclus-related polyphosphate-accumulating organism (PAO) (Accumulibacter) existed in the enhanced biological phosphorus removal system and was not detected in the A^2/O process. Compared with the A^2/O process, the A/O-sequencing batch reactor dephosphorization process was more suitable for the enrichment of phosphorus accumulating microorganisms (Accumulibacter), and the abundance of Accumulibacter was relatively high. In the A^2/O process, the phosphorus accumulating microorganisms competed with the denitrification microorganisms, which was reflected in

Table 4 | Relative abundance of functional bacteria genera in three phases

Functional bacteria	Phylum	Genus	Relative abundance/% Phase I	Phase II	Phase III
AOB	Proteobacteria	*Nitrosomonas*	0.22–0.24	0.10–0.14	0.01–0.02
NOB	Nitrospirae	*Nitrospira*	5.34–6.62	2.90–3.26	2.17–2.65
DNB	Proteobacteria	*Azospira*	1.92–2.12	19.96–23.81	9.77–10.83
	Proteobacteria	*Thauera*	1.48–1.58	0.93–1.15	1.76–2.00
	Proteobacteria	*Dokdonella*	0.09–0.12	0.03–0.04	1.76–1.91
	Proteobacteria	*Thiothrix*	0.01–0.02	0.01–0.02	0.92–1.15
	Proteobacteria	*Pseudomonas*	0.32–0.46	0.03–0.06	0.01–0.03
	Proteobacteria	*Zoogloea*	0.73–0.85	0.06–0.07	0.57–0.66
	Proteobacteria	*Acinetobacter*	0.47–0.91	0.12–0.17	0.22–0.41
	Bacteroidetes	*Terrimonas*	1.75–1.94	0.21–0.27	0.03–0.05
	Bacteroidetes	*Phaeodactylibacter*	0.94–1.02	0.61–0.68	1.92–1.99
	Bacteroidetes	*Ferruginibacter*	2.04–2.25	0.07–0.09	0.70–0.87
	Planctomycetes	*Tepidisphaera*	0.83–0.88	0.81–1.04	1.10–1.46
PAOS	Proteobacteria	*Dechloromonas*	2.81–3.02	2.70–3.01	1.32–1.86
	Proteobacteria	*Acinetobacter*	0.47–0.91	0.22–0.41	0.04–0.05
	Proteobacteria	*Povalibacter*	0.89–0.94	1.97–2.12	2.90–4.01
	Bacteroidetes	*Chryseolinea*	0.86–0.92	1.17–1.38	2.22–2.58
	Planctomycetes	*Pirellula*	0.34–0.39	1.11–1.53	1.57–2.08
	Proteobacteria	*Pseudomonas*	0.09–0.13	0.32–0.46	0.69–0.87
GAOS	Proteobacteria	*Defluviimonas*	0.25–0.32	0.15–0.19	0.23–0.32

the change of species and abundance of the functional microbial community (Wang *et al.* 2017). As a result, the relative abundance of six genera relevant to phosphorus removal in the system such as *Dechloromonas*, *Acinetobacter*, *Povalibacter*, *Chryseolinea*, *Pirellula* and Pseudomonas showed obvious rules with the experiment in progress. The relative abundances of *Dechloromonas* and *Acinetobacter* decreased, however the relative abundances of *Povalibacter*, *Chryseolinea* and *Pirellula* increased with the increasing in organic loading. Meanwhile lower levels of Defluviicoccus-related bacteria considered as typical GAOs were detected in the study.

Correlation between microbial community behavior and environmental variables

PCA was conducted to elucidate the potential relationship between microbial composition and key environmental variables as shown in Figure 5. It can be seen from the analysis results that *Nitrospira*, Terrimonas, Pseudomonas and Defluviimonas were strongly correlated with phase I, which indicated that low organic loading would promote the growth of these genera. Tepidisphaera was significantly correlated with the PS and EPS, indicating that Tepidisphaera played a crucial role in promoting the secretion of EPS and PS. *Pirellula*, *Povalibacter*, *Chryseolinea*, Phaeodactylibacter, Thauera and Tepidisphaera were correlated with

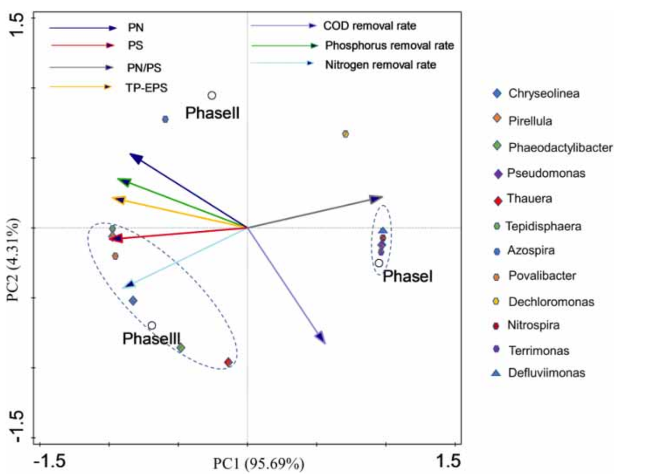


Figure 5 | PCA based on the sequencing data and environmental variables. Arrows indicate the direction and magnitude of environmental variables associated with microbial communities. The length of an arrow-line indicates the strength of the relationship between the environmental variable and microbial community. Each diamond or hexagon represents individual bacterial genus of one sludge sample.

phase III, which indicated that high organic loading would promote the growth of these genera. Meanwhile, PS was positively correlated with both denitrification and phosphorus removal, presumably because PS can be used by microorganisms as an organic carbon source for denitrification and phosphorus removal. The organic loading significantly affected the microbial community, which was in accordance with previous studies that demonstrated that the influent organic loading was the main variable shaping the bacterial community (Fan *et al.* 2019; Cao *et al.* 2020).

CONCLUSION

This study revealed that increasing influent organic loading significantly improved the performance of the A^2/O continuous-flow system. Meanwhile, the secretion of EPS was promoted, and PS was positively correlated with nitrogen and phosphorus removal.

Phosphorus in sludge mainly existed in the form of IP, which mainly existed in the form of AP. The change of TP content in EPS was mainly caused by the change in IP content, while the change of IP content in EPS was mainly caused by the change of NAIP content, which played an important role in the extracellular phosphorus removal.

There was a strong mapping relationship between the treatment function of the reactor and the functional flora distribution of the microorganism. The abundance of *Nitrosomonas* and *Nitrospira* decreased with the increase in organic loading. The group of denitrifiers was large, and *Azospira* was the most abundant genus among them. *Dechloromonas*, *Acinetobacter*, *Povalibacter*, *Chryseolinea* and *Pirellula* were the functional genera closely associated with phosphorus removal.

ACKNOWLEDGEMENTS

The study was financially supported by Tianjin Education Commission scientific research project (2016CJ09), the National Natural Science Foundation of China (No.51678388), and Key project of Tianjin Natural Science Foundation (18JCZDJC10080).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Cao, J., Zhang, T., Wu, Y., Sun, Y., Zhang, Y., Huang, B., Fu, B., Yang, E., Zhang, Q. & Luo, J. 2020 Correlations of nitrogen removal and core functional genera in full-scale wastewater treatment plants: influences of different treatment processes and influent characteristics. *Bioresource Technology* **297**, 1–10. doi:10.1016/j.biortech.2019.122455.

Fan, Z., Zeng, W., Wang, B., Chang, S. & Peng, Y. 2019 Analysis of microbial community in a continuous flow process at gene and transcription level to enhance biological nutrients removal from municipal wastewater. *Bioresource Technology* **286**, 1–9. doi:10.1016/j.biortech.2019.121374.

Geyik, A. G. & Çeçen, F. 2015 Variations in extracellular polymeric substances (EPS) during adaptation of activated sludges to new feeding conditions. *International Biodeterioration & Biodegradation* **105**, 137–145. doi:10.1016/j.ibiod.2015.08.021.

Geyik, A. G. & Çeçen, F. 2016 Production of protein- and carbohydrate-EPS in activated sludge reactors operated at different carbon to nitrogen ratios. *Journal of Chemical Technology & Biotechnology* **91** (2), 522–531. doi:10.1002/jctb.4608.

Gong, Y. K., Zhao, Q. & Peng, Y. Z. 2019 Variation of N_2O emission and EPS production during simultaneous nitrification and denitrification in SBBR under different C/N ratio. *CIESC Journal* **70** (12), 4847–4855. doi: 10.11949/0438-1157.20190753.

Guo, C., Liu, H. Y., Wang, Q., Liu, S. Q., Luo, Y. L. & Liu, X. Y. 2015 Existing morphology of phosphorus in dephosphorization granular sludge and its content analysis. *Safety and Environmental Engineering* **22**, 44–48. doi:10.13578/j.cnki.issn.1671-1556.2015.02. 009.

Guo, J., Ni, B.-J., Han, X., Chen, X., Bond, P., Peng, Y. & Yuan, Z. 2017 Unraveling microbial structure and diversity of activated sludge in a full-scale simultaneous nitrogen and phosphorus removal plant using metagenomic sequencing. *Enzyme and Microbial Technology* **102**, 16–25. doi:10.1016/j.enzmictec.2017.03.009.

Lan, X. 2020 *Study on Optimization of A2/O Process Operation in Urban Wastewater Treatment Plant*. Dissertation, Hebei University of Engineering.

Li, Z. 2019 *Metabolism Characteristics of Polyphosphate Accumulating Organisms and Quorum Sensing of Granular Sludge in A/O-SBR System at Low Temperature*. Dissertation, Tianjin Chengjian University.

Li, W. W., Zhang, H. L., Sheng, G. P. & Yu, H. Q. 2015 Roles of extracellular polymeric substances in enhanced biological phosphorus removal process. *Water Research* **86**, 85–95. doi:10.1016/j.watres.2015.06.034.

Li, D., Lv, Y. F., Zeng, H. P. & Zhang, J. 2016 Long term operation of continuous-flow system with enhanced biological phosphorus removal granules at different COD loading. *Bioresource Technology* **216**, 761–767. doi:10.1016/j.biortech.2016.06.022.

Li, X., Yang, W. L., He, H., Wu, S., Zhou, Q., Yang, C., Zeng, G., Luo, L. & Lou, W. 2018 Responses of microalgae Coelastrella sp. to stress of cupric ions in treatment of anaerobically digested swine wastewater. *Bioresource Technology* **251**, 274–279. doi:10.1016/j.biortech. 2017.12.058.

Li, Y. J., Wang, S. P., Li, Z. & Sun, L. P. 2020 Extraction method and structural and composition characteristics of extracellular polymeric substances in granular sludge from an enhanced biological phosphorus removal system. *Desalination and Water Treatment* **185**, 41–50. doi:10.5004/dwt.2020.25389.

Long, X. Y., Tang, R. & Fang, Z. D. 2017 The roles of loosely-bound and tightly-bound extracellular polymer substances in enhanced biological phosphorus removal. *Chemosphere* **189**, 679–688. doi:10.1016/j.chemosphere.2017.09.067.

Massara, T. M., Malamis, S., Cuisasola, A., Noutsopoulos, C. & Katsou, E. 2017 A review on nitrous oxide (N_2O) emissions during biological nutrient removal from municipal wastewater and sludge reject water. *Science of Total Environment* **596–597**, 106–123. doi:10.1016/j.scitotenv.2017.03.191.

Rong, Y., Liu, X., He, Y., Zhang, W. & Jin, P. 2019 Enhanced nutrient removal and microbial community structure in a step-feed A^2/O process treating low C/N municipal wastewater. *Environmental Science* **40** (09), 4113–4120. doi:10.13227/j.hjkx.201903192.

Rusanowska, P., Cydzik-Kwiatkowska, A., Świątczak, P. & Wojnowska-Baryła, I. 2019 Changes in extracellular polymeric substances (EPS) content and composition in aerobic granule size-fractions during reactor cycles at different organic loads. *Bioresource Technology* **272**, 188–193. doi:10.1016/j.biortech.2018.10.022.

Sheng, G. P., Yu, H. Q. & Li, X. Y. 2010 Extracellular polymeric substances (EPS) of microbial aggregates in biological wastewater treatment systems: a review. *Biotechnology Advances* **28**, 882–894. doi:10.1016/j.biotechadv.2010.08.001.

Shi, Y. H., Huang, J. H., Zeng, G. M., Gu, Y. L., Chen, Y. N., Hu, Y., Tang, B., Zhou, J. X., Yang, Y. & Shi, L. X. 2017 Exploiting extracellular polymeric substances (EPS) controlling strategies for performance enhancement of biological wastewater treatments: an overview. *Chemosphere* **180**, 396–411. doi:10.1016/j.chemosphere.2017.04.042.

Szabo, E., Liébana, R. & Hermansson, M. 2017 Microbial population dynamics and ecosystem functions of anoxic/aerobic granular sludge in sequencing batch reactors operated at different organic loading rates. *Frontiers in Microbiology* **8**, 1–14. doi:10.3389/fmicb. 2017.00770.

Tan, C. H., Kon, K. S., Xie, C., Tay, M., Zhou, Y., Williams, R., Ng, W. J., Rice, S. A. & Kjelleberg, S. 2014 The role of quorum sensing signaling in EPS production and the assembly of a sludge community into aerobic granules. *ISME Journal* **8**, 1186–1197. doi:10.1038/ismej.2013.240.

Wang, Y., Nan, Y., Yuan, L. & Chen, M. 2017 Comparative analysis of microbial population structure of A^2/O and SBR system. *Industrial Microbiology* **47** (6), 25–30. doi:10.3969/j.issn.1001-6678.2017.06.005.

Wang, H., Song, Q., Wang, J., Zhang, H., He, Q., Zhang, W., Song, J., Zhou, J. & Li, H. 2018 Simultaneous nitrification, denitrification and phosphorus removal in an aerobic granular sludge sequencing batch reactor with high dissolved oxygen: effects of carbon to nitrogen ratios. *Science of the Total Environment* **642**, 1145–1152. doi:10.1016/j.scitotenv.2018.06.081.

Wang, X., Chen, Z., Shen, J. & Zhao, X. 2019 Impact of carbon to nitrogen ratio on the performance of aerobic granular reactor and microbial population dynamics during aerobic sludge granulation. *Bioresource Technology* **271**, 258–265. doi:10.1016/j.biortech.2018.09.119.

Yang, S. S., Pang, J. W., Guo, W. Q., Yang, X. Y., Wu, Z. Y., Ren, N. Q. & Zhao, Z. Q. 2017 Biological phosphorus removal in an extended ASM2 model: roles of extracellular polymeric substances and kinetic modeling. *Bioresource Technology* **232**, 412–416. doi:10.1016/j.biortech.2017.01.048.

Yu, S., Sun, P., Zheng, W., Chen, L., Zheng, X., Han, J. & Yan, T. 2014 The effect of COD loading on the granule-based enhanced biological phosphorus removal system and the recoverability. *Bioresource Technology* **171**, 80–87. doi:10.1016/j.biortech.2014.08.057.

Zhang, S., Huang, Z., Lu, S., Zheng, J. & Zhang, X. 2017 Nutrients removal and bacterial community structure for low C/N municipal wastewater using a modified anaerobic/anoxic/oxic (mA^2/O) process in North China. *Bioresource Technology* **243**, 975–985. doi:10.1016/j.biortech.2017.07.048.

Zhang, M., Wang, Y., Fan, Y., Liu, Y., Yu, M., He, C. & Wu, J. 2020 Bioaugmentation of low C/N ratio wastewater: effect of acetate and propionate on nutrient removal, substrate transformation, and microbial community behavior. *Bioresource Technology* **306**, 1–13. doi:10.1016/j.biortech.2019.122465.

Zhao, W. H., Huang, Y., Wang, M. X., Pan, C., Li, X., Peng, Y. & Li, B. 2018 Post-endogenous denitrification and phosphorus removal in an alternating anaerobic/oxic/anoxic (AOA) system treating low carbon/nitrogen (C/N) domestic wastewater. *Chemical Engineering Journal* **339**, 450–458. doi:10.1016/j.cej.2018.01.096.

Zheng, L., Ren, M., Xie, E., Ding, A., Liu, Y., Deng, S. & Zhang, D. 2019 Roles of phosphorus sources in microbial community assembly for the removal of organic matters and ammonia in activated sludge. *Frontiers in Microbiology* **10**, 1–13. doi:10.3389/fmicb.2019.01023.

Zhou, J., Zhou, L. X. & Huang, H. Z. 2013 Optimization of extracellular polymeric substance extraction method and its role in the dewaterability of sludge. *Environmental Science* **34**, 2752–2757. doi:10.13227/j.hjkx.2013.07.056.

First received 24 December 2020; accepted in revised form 12 April 2021. Available online 26 April 2021