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► To cite this version:

Lihui Cui, Hao Shen, Pengfei Kang, Xiaoying Guo, Haisong Li, et al.. Stability and nutrients removal performance of a Phanerochaete chrysosporium-based aerobic granular sludge process by step-feeding and multi A/O conditions. *Bioresource Technology*, 2021, 341, pp.125839. 10.1016/j.biortech.2021.125839 . hal-04291551

HAL Id: hal-04291551

<https://unilim.hal.science/hal-04291551v1>

Submitted on 26 Jun 2024

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1 **Stability and nutrients removal performance of a *Phanerochaete***
2 ***chrysosporium*-based aerobic granular sludge process by**
3 **step-feeding and multi A/O conditions**

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17

18 **Abstract**

19 A *Phanerochaete chrysosporium*-based aerobic granular sludge (PC-AGS) was
20 developed by inoculating fungal mycelial pellets into a lab-scale aerobic granular
21 sequencing batch reactor (AGSBR). A strategy using step-anaerobic feeding coupled
22 with multi A/O conditions was adopted. The results showed that the removal
23 efficiencies for total phosphorus (TP) and total inorganic nitrogen (TIN) were $94.56 \pm$
24 2.92% and $75.20 \pm 7.74\%$, respectively, under relatively low aeration time. Compared
25 with original AGS, the content of extracellular proteins for PC-AGS obviously
26 increased from 18.61 to 41.44 mg/g MLSS by the end of phase I. Moreover, the
27 mature granules had a larger size and better stability during the 100 days operation.
28 Furthermore, the analysis of microbial diversity detected many key functional groups
29 in PC-AGS granules that were beneficial to nutrients removal. This work
30 demonstrated that the addition of fungal pellets not only enhanced the removal
31 performance, but also improved the stability of the AGS system.

32

33 **Keywords:** Aerobic granular sludge; Mycelium pellets; Nutrients removal; Stability

34

35 **1. Introduction**

36 Since aerobic granular sludge (AGS) technology was firstly reported in 1991, the
37 formation mechanisms and application of aerobic granular sludge have become hot
38 topics in the field of water treatment research (Mishima & Nakamura, 1991; Zhang et
39 al., 2020). Compared with traditional activated sludges, AGS exhibits a denser
40 structure, quicker settlement, higher pollutant removal performance, greater biomass
41 enrichment, and higher resistance to load shock (Yan et al., 2019; Yuan et al., 2019).
42 However, the main problems encountered when utilizing AGS is its instability, which
43 limits its application in the wastewater treatment. The phenomenon of aerobic
44 granular sludge disintegration was often reported after long-term operation, which
45 leads to subsequent biomass loss and the final failure of AGS system (Liu et al., 2004;
46 Schwarzenbeck et al., 2005). To address this instability, biological process with the
47 potential to enhance granular stability and pollutant removal performance must be
48 investigated.

49 Many studies aimed at improving the stability of AGS have been conducted, but most
50 have focused on organic loading rate (OLR), C/N ratio, hydraulic selection pressure,
51 substrate composition, temperature, and pH as determining factors (Corsino et al.,
52 2017; Wang et al., 2018a; Yin et al., 2019a; Yuan et al., 2019). However, in actual
53 practice, the operating conditions of reactors and characteristics of wastewater are
54 difficult to adjust and change in real time. So, rather than actively responding to
55 reactor or wastewater conditions, microbial attachment growth strategies have been
56 applied as effective methods to decrease sludge loss and increase biomass production,
57 which enhances sludge stability by adding biomass carriers (Wang et al., 2019; Zhang
58 et al., 2020). In fact, mycelial pellets are a novel and eco-friendly biomass material
59 that can combine other organisms and substances to form self-immobilized

60 bio-mixtures that have a good self-immobilization capacity, making them suitable
61 biomass carriers for promoting aerobic granulation (Wang et al., 2019). Compared
62 with other synthesized carriers, mycelial pellets could efficiently adsorb or remove
63 pollutants from wastewater through physic-chemical reaction and biodegradation
64 while not creating secondary pollution (Espinosa-Ortiz et al., 2016). Many studies
65 have confirmed that fungi serve certain functions in sludge treatments, like
66 degradation of refractory organic pollutants, nutrients (nitrogen and phosphorus)
67 removal and can become fungal pellets (Dalecka et al., 2020; Espinosa-Ortiz et al.,
68 2016). *Phanerochaete chrysosporium*, an infamous white rot fungus, has a high
69 biosorption capacity and can produce enzymes that degrade various refractory and
70 toxic organic compounds (Lu et al., 2009). Furthermore, it possesses an abundance of
71 pores and a large specific surface area, which are conducive to the transfer of
72 materials and oxygen (Wang et al., 2014). Although the mycelial pellets technology
73 has good potential in wastewater treatment, especially for the removal of aromatic
74 compounds (e. g. lignin), the real application of *Phanerochaete chrysosporium*
75 mycelial pellets is rarely reported. Considering that the growth of bacteria is more
76 competitive than that of fungi when treating real wastewater, it is necessary to
77 investigate the influence of mycelial pellets on the classical AGS system.

78 Therefore, our study aimed to develop a strategy for the fast establishment of
79 *Phanerochaete chrysosporium*-based aerobic granular sludge (PC-AGS) in a
80 sequencing batch reactor (SBR) by inoculating white rot fungi *Phanerochaete*
81 *chrysosporium* mycelium pellets into AGS. Meanwhile, the characteristics,
82 performance, extracellular polymeric substances (EPS) content, and functional
83 microorganisms of the mature granular consortia were observed over 100 days
84 post-inoculation so as to clarify the possible mechanisms underlying this strategy. The

85 results from this study will help establish a feasible, eco-friendly, and simple strategy
86 for full-scale applications.

87 **2. Materials and methods**

88 **2.1. Preparation of mycelial pellets**

89 *Phanerochaete chrysosporium* was inoculated into 100 mL liquid medium (mg/L)
90 which consisted of glucose (10,000), ammonium tartrate (200), KH_2PO_4 (2000),
91 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (750), CaCl_2 (100), CuSO_4 (64), and TWEEN 80 (1000). Additionally,
92 20 mL of a trace element solution was added into 1000 mL of medium. The trace
93 element solution (mg/L) contains: MgSO_4 (3000), MnSO_4 (500), 1 g/L NaCl (1000),
94 0.1 g/L $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (100), CoCl_2 (100), $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ (10), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (100),
95 $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (10), and H_3BO_3 (10). *Phanerochaete chrysosporium* with prepared
96 medium were incubated at 30°C with a constant rotary speed (150 rpm) for around 5
97 days until numerous mycelial pellets were formed.

98 **2.2. AGS and synthetic wastewater composition**

99 The selected original AGS (the mean size was 2650 μm) were cultivated in laboratory
100 for more than 200 days. The initial concentration and the sludge volume index (SVI_{30})
101 of AGS in this experiment was approximately 7000 mg/L and 28 mL/g, respectively.
102 The composition of the prepared synthetic wastewater (Table 1) included three
103 organic carbon sources and each carbon source contributed 33% of the total COD
104 (1000 ± 50 mg/L).

105 **2.3. Reactor setup and operation**

106 The experimental set-up consisted of a bioreactor with a working volume of 2 L
107 (internal diameter = 5 cm and total height = 120 cm). The SBR was automatically

108 maintained at $25 \pm 1^\circ\text{C}$. The influent was fed into the bottom of the reactor. The SBR
109 was operated in 6 h cycles, each of which included three sub-cycles ($115 \times 3 = 445$
110 min), the settling (5 min) and the discharging (10 min). Every internal cycle (115 min)
111 included 10 min of anaerobic feeding with 0.23 L wastewater, 45 min of anaerobic and
112 60 min of aeration. As shown in Table 2, the experimental period was divided into
113 three phases (phase I, II, and III) according to the various nitrogen and phosphorus
114 loading rates. Approximately 2 g (wet weight) of mycelial pellets with diameters
115 ranging between 1–3 mm was added into the reactor at the start of each of the three
116 phases.

117 **2.4. Analytical methods**

118 Water samples were regularly collected from the SBR system. The samples were
119 immediately filtered through a $0.45 \mu\text{m}$ filter membrane and stored at 4°C . Chemical
120 oxygen demand (COD), ammonia ($\text{NH}_4^+\text{-N}$), nitrite ($\text{NO}_2^-\text{-N}$), nitrate ($\text{NO}_3^-\text{-N}$),
121 phosphate ($\text{PO}_4^{3-}\text{-P}$), sludge volume index after 30 (SVI₃₀) and 5 (SVI₅) minutes,
122 MLSS, and MLVSS were measured in accordance with standard methods (APHA,
123 2005). Total inorganic nitrogen (TIN) was computed as the sum of $\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$,
124 and $\text{NO}_3^-\text{-N}$ concentrations (Yan et al., 2019). The particle sizes of randomly selected
125 granules (at least 100 granules for each sampling) were measured by the software of
126 SEM image analysis (Nano Measurer System, 1.2.5). The morphology of the granular
127 sludge samples was observed by digital camera and scanning electron microscopy
128 (SEM, Zeiss/Auriga-bu, Germany). The aerobic granules were gathered from the
129 bottom of SBR, then immediately washed three times with distilled water and dried at
130 105°C for 24 h. After sulfuric acid-hydrogen peroxide digestion, the content of the
131 total phosphorus (TP) was measured in accordance with standard methods (APHA,
132 2005). A heat extraction was applied to extract the EPS of the granular sludge

133 (Morgan et al., 1990). Then the concentration of polysaccharides (PS) and the protein
134 (PN) in the EPS were quantified via the phenol-sulfuric acid method and the Lowry
135 method (Dubios et al., 1956; Lowry et al., 1951).

136 **2.5. Microbial community analysis**

137 Sludge samples were extracted from the SBR reactor on days 34, 66, and 99, denoted
138 as G1, G2, G3, respectively. DNA extraction, PCR amplification, and pyrosequencing
139 were conducted sequentially using the general primers 338F
140 (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R
141 (5'-GGACTACHVGGGTWTCTAAT-3') for bacteria following previously
142 established procedures (Cheng et al., 2020). High-throughput sequencing was
143 performed by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China) using the
144 MiSeq PE300 platform (Illumina, USA) according to the manufacturer's instructions.
145 The bioinformatics analysis was performed according to previously described
146 methods (Cheng et al., 2020).

147 **3. Results and discussion**

148 **3.1. Characterization of PC-AGS**

149 **3.1.1 Changes in morphology**

150 The seed sludge was mainly characterized by a smooth outer surface with regular and
151 dense structures with a mean size of 2650 μm . In phase II (100 mg/L $\text{NH}_4^+\text{-N}$), white
152 filamentous organisms were observed growing on the surface of some granules. The
153 aerobic granules with larger sizes usually had higher biomasses and richer microbial
154 communities, resulting in an improved pollutant removal performance (Cui et al.,
155 2019). However, the structures of large size aerobic granules were often unstable
156 owing to the growth of filamentous bacterial (Xu et al., 2020). After phase II, the

157 biomass decreased rapidly due to the cracking and disintegration of granules, but the
158 fragments were still large enough to be left in the reactor as a carrier for particle
159 formation, as was also noted by [Wang et al. \(2014\)](#). At the same time, the white
160 mycelial pellets with smooth surfaces roughened and turned gray after being
161 inoculated into the reactor, probably owing to the adhesion of flocs. At the initial
162 stage with the addition of MPs, two kinds of granular sludge including the original
163 granular sludge and the granular sludge dominated by fungi could be easily separated
164 in the system, while these two types eventually become one type of granular sludge
165 named PC-AGS in the system at the steady period. PC-AGS had a dense structure that
166 inhibited filamentous overgrowth under alkaline conditions, and a large number of
167 intertwined filaments occupied the surface with many adsorbed cocci and bacilli. In
168 fact, many cocci and bacilli cells could be attached to the surface and interior of MPs
169 and some hyphae could adhere to the surface of granular sludge to promote the
170 formation of microstructure pores or channels in the granules ([Geng et al., 2020](#);
171 [Geng et al., 2021](#)). Therefore, PC-AGS provided more surface area to absorb
172 suspended solids and had many channels which could facilitate the transfer of
173 nutrients and oxygen.

174 **3.1.2 Biomass retention and granular settleability**

175 MLSS and SVI are important parameters for evaluating the stability of AGS system.
176 In phase I, due to disintegration, the flocculent sludge aggregated in PC mycelium
177 pellets to form PC-AGS, and the MLSS had reached 9.50 g/L by day 34 (Fig. 1A).
178 Previous research has reported that the microbial growth dominated by specific
179 microorganisms was responsible for sludge granulation, and that the enhanced
180 aggregation of microorganisms inside the flocs led to the formation of compact
181 particles ([Barr et al., 2010](#)). The increases in influent nitrogen and phosphorus

182 concentration during phase II only resulted in decreases in the observed MLSS and
183 MLVSS concentrations of PC-AGS between days 43 and 57 (from 12.60 and 8.05 g/L
184 to 5.66 and 3.34 g/L for MLSS and MLVSS, respectively). The growth of filamentous
185 fungi and bacteria was the primary reason for the change in biomass, and free fungi
186 and flocs were washed away with the discharged effluents. Thereafter, after the
187 microorganism had adapted to the changed environment, the MLSS and MLVSS
188 concentrations of the PC-AGS continuously increased, gradually recovering to even
189 higher levels despite the decreased influent ammonium concentration in phase III. In
190 fact, the previous study showed that the classical AGS system without the addition of
191 PCs was fragile and instable under step-feeding and multi A/O conditions (Cheng et
192 al., 2020). Similarly, Geng et al. (2021) observed a loss of morphological integrity and
193 deterioration of settling properties of the granules during prolonged operation without
194 adding MPs. This indicated that using a microbial attachment growth strategy could
195 greatly decrease sludge loss and increase biomass production for an AGS system
196 (Zhang et al., 2020). During operation, the MLVSS/MLSS ratios decreased gradually
197 (from 0.70 to 0.55), and were lower than those reported by Yin et al. (2019b). This
198 low ratio was possibly due to the operational conditions (without regular discharge of
199 the excess sludge for the PC-AGS system) and the abundant inorganic substances in
200 the synthetic wastewater.

201 In contrast, a declining trend was observed for the SVI during the operation (Fig. 1B),
202 indicating that the granular settleability of the PC-AGS system was gradually
203 improving. SVI_{30} and SVI_5 decreased to their minimums (20.27 and 21.08 mL/g,
204 respectively) on the 36th day, before they slightly increased, likely because of the
205 decrease in MLSS that probably resulted from the competition between fungi and
206 bacteria for nutrients. However, under operating conditions, SVI_{30} and SVI_5 recovered

207 rapidly and eventually stabilized in phase III at about 17 and 18 mL/g, respectively.
208 Moreover, the SVI values were always less than those of typical aerobic granules
209 which means the better settleability of PC-AGS (Liu & Tay, 2004). The overall
210 SV₃₀/SV₅ profile was increasing (Fig. 1B), and the maximum reached to 96% on the
211 36th day. Then, it stabilized above 90% for the remainder of the operational time,
212 which implied the stability of PC-AGS was better than traditionally AGS.

213 3.1.3. Changes in EPS

214 EPS secreted by microorganisms is mainly composed of PN and PS and is essential to
215 binding microbial aggregates together and guaranteeing the structural stability of
216 granules (Franca et al., 2018). The PS and PN contents and the ratios of PN/PS of the
217 three phases are shown in Fig. 1C. The initial PN content of the inoculated sludge was
218 low, but it gradually increased and eventually stabilized. These results were consistent
219 the previous report that revealed that PN changed the surface properties of granules
220 and promoted compact and stable aggregate structures (Yan et al. 2019). During the
221 phase II, the PN content obviously decreased when the ratio of COD:N was decreased
222 from 100:5 to 100:10, resulting in the PN/PS dropping from 2.53 to 1.58. The
223 decrease for the PN/PS ratio reflected the less negative charge and higher cell surface
224 hydrophobicity, which could probably result in increased instability of PC-AGS
225 (Zhang et al., 2007). Then, the flocculent sludge produced by the wastewater
226 gradually was aggregated to form new granules with the help of mycelial pellets,
227 which might be the reason for the increased EPS production in phase III (from 35.91
228 to 45.16 mg/g MLSS). The results of the EPS content analysis were consistent with
229 the changes in physical characteristics of the PC-AGS, which confirmed the
230 relationship between EPS content and granule structure proposed in previous studies
231 (Corsino et al., 2017). On the other hand, it should be pointed out that from the

232 Day-32 the PS content stabilized at 15.14 ± 1.58 mg/g MLSS, probably owing to the
233 great quantity of PS content (41.28 ± 4.91 mg/g MLSS) secreted by *Phanerochaete*
234 *chrysosporium*. It has been reported that PS may play a crucial role in microbial
235 resistance to external pressure, and therefore, also in maintaining AGS stability (Xu et
236 al., 2020; Yin et al., 2019a). Filamentous fungi can secrete a polymeric extracellular
237 matrix (exopolysaccharides) that allows a growing colony to tenaciously adhere to
238 substrates, owing to the formation of strong and multilayered coacervates (Harding et
239 al., 2009). Furthermore, on day 69 in this study, the PS content in the outer layer of
240 the PC-AGS was higher than that of the inner layer (25.86 ± 2.56 and 10.41 ± 0.84
241 mg/g MLSS, respectively), which greatly enhanced the granule strength (McSwain et
242 al. 2005).

243 **3.2. Performance of PC-AGS system**

244 **3.2.1. C, N, and P removal performance**

245 COD and nutrients (nitrogen and phosphorus) removal efficiencies were analyzed
246 during three phases (Fig. 2). PC-AGS contributed to high COD and phosphorus
247 removal efficiencies ($> 80\%$ and $> 90\%$, respectively) in phase I (Fig. 2A and B). The
248 removal efficiencies of nitrogen and phosphorus for PC-AGS system showed an
249 obvious decreasing trend when their influent concentrations increased to about 100
250 and 20 mg/L, respectively, which might have been related to the decrease in MLSS
251 concentration. After a short period of adaptation, the removal efficiencies of COD and
252 phosphorus of PC-AGS were increased to 94.37% and 90.13%, respectively, possibly
253 due to the enhanced biomass retention. After 100 days operation, the average
254 phosphorus content of PC-AGS ($4.75 \pm 0.08\%$, m/m) was higher than that of the
255 conventional activated sludge, which was probably due to the accumulation of

256 phosphorus as the forms of hydroxyapatite (HAP) in the core of aerobic granules
257 (Zhang et al. 2013). In general, the changes in the COD and $\text{PO}_4^{3-}\text{-P}$ removal
258 efficiencies were consistent with the variations in MLSS and MLVSS. It has been
259 reported that mycelial pellets can combine with other substances and organisms to
260 form compact particles and remove pollutants from wastewater via biosorbence and
261 biodegradation (Espinosa-Ortiz et al., 2016). In addition, Dalecka et al. (2020)
262 claimed that white rot fungi was able to remove phosphorus by biosorption and
263 metabolic activity. Therefore, PC-AGS might improve COD and $\text{PO}_4^{3-}\text{-P}$ removal by
264 improving the environment for microorganisms.

265 The removal efficiencies of ammonium and TIN were monitored throughout the
266 long-term experiment. Initially, the removal efficiencies of ammonium and TIN were
267 low, and the effluent ammonium was above 17 mg/L on the first day (Fig. 2C). This
268 might have been because the sludge had not adapted to the new microenvironment.
269 Furthermore, the ammonium and TIN removal efficiencies in the PC-AGS system
270 were clearly affected by the C/N ratio. The influent had a constant COD concentration
271 (around 1000 mg/L) and the COD/N ratios of 20, 10, and 20 during the three phases,
272 which resulted in average effluent ammonium concentrations of 8.35 ± 3.03 , $57.80 \pm$
273 11.23 , and 13.28 ± 3.56 mg/L, corresponding to removal efficiencies of $83.39 \pm$
274 6.00% , $43.40 \pm 10.92\%$, and $73.43 \pm 6.97 \%$, respectively. These trends may be
275 explained by the relatively high free-ammonia (FA) concentrations, which may have
276 inhibited microbial activity. With the decrease in $\text{NH}_4^+\text{-N}$ removal efficiency, the
277 granular structure was also adversely affected. Yang et al. (2004) observed that
278 aerobic granulation was negatively influenced when the concentration of FA was
279 more than 23.5 mg/L and the respiratory activity of nitrifying and heterotrophic
280 bacteria was reduced as FA risen from 2.5 to 39.6 mg/L. In addition, almost no

281 accumulation of nitrate or nitrite was observed for the duration of reactor operation
282 (effluent nitrate and nitrite concentrations were less than 1.00 mg/L), indicating the
283 removal rate of TIN was close to that of $\text{NH}_4^+\text{-N}$. Apparently, the alternation between
284 aerobic and anaerobic conditions improved the denitrification process and achieved a
285 much better TIN removal efficiency (Yuan et al., 2019). The phenomenon indicated
286 that decreasing COD/N ratios had a harmful effect on ammonium removal, but had no
287 influence on nitrate or nitrite removal in the PC-AGS system.

288 **3.2.2. Kinetic study**

289 In order to further analyze the mechanisms by which carbon, nitrogen, and
290 phosphorus were simultaneously removed by PC-AGS, cyclic performance was
291 examined at the end of each phase (Fig. 3). Compared with previous study (Gao et al.,
292 2020), COD was not completely consumed until the end of the anaerobic stage, and
293 then it decreased in the aerobic stage. This implied that COD was absorbed by
294 anaerobic microorganisms to facilitate the phosphorus release in the anaerobic stage
295 and denitrification in both anaerobic and aerobic stages. It is worth noting that $\text{PO}_4^{3-}\text{-P}$
296 was released in the anaerobic phase, but then absorbed during the subsequent aerobic
297 phase by polyphosphate accumulating organisms (PAOs). This process resulted in the
298 $\text{PO}_4^{3-}\text{-P}$ being removed from the system by the end of the aerobic cycle, implying that
299 the PC-AGS had a stable enhanced biological phosphorus removal (EBPR) phenotype
300 (Zhang et al., 2013). The extended anaerobic stage ensured efficient phosphorus
301 removal, which may be the primary reason that the step feeding method had such an
302 excellent and stable phosphorus removal efficiency (Wang et al., 2018a). In the
303 meantime, the concentrations of phosphorus were higher in phase II and phase III
304 (from 10.22 mg/L in phase I to 19.66 and 19.79 mg/L in phase II and phase III,
305 respectively) matching the increase in influent phosphorus concentration, implying

306 that the activity of PAOs had been enhanced. This showed that the main mechanism
307 of phosphorus removal was biological phosphorus removal, and PC-AGS had higher
308 PAO activity when the step feeding strategy was applied.

309 Regarding nitrogen, the influent ammonium concentration was slightly delayed after
310 each feeding in phase II and phase III compared with phase I. This might have been
311 because a portion of the ammonium was initially absorbed by PC-AGS, but was
312 desorbed from the granules during feeding, which was consistent with the previous
313 results that showed a large portion of influent ammonium (30-40%) adsorbed by AGS
314 ([Bassin et al., 2011](#)). The PC-AGS exhibited a unique structure with channels and a
315 relatively large specific surface area, which means it contained an abundance of
316 interior adsorption sites ([Wang et al., 2018b](#)). However, the oxidation rate of
317 ammonium was remarkably low in the aerobic stage, which was possibly due to low
318 ammonia oxidizing bacteria (AOB) activity. Filamentous microorganisms can
319 consume large proportions of the DO, which might have an inhibitory effect on the
320 growth of AOB ([Cheng et al., 2020](#)). Meanwhile, the nitrite and nitrate concentrations
321 were very low throughout the whole cycle, but especially in phase II (< 0.5 mg/L),
322 thus demonstrating the excellent removal rate for TIN. An overall nitrogen loss
323 occurred since the accumulated nitrite and nitrate were less than the removed $\text{NH}_4^+\text{-N}$,
324 indicating that nitrification and denitrification (SND) occurred simultaneously in the
325 PC-AGS system ([He et al., 2020](#)). Furthermore, the alternating A/O condition with
326 step feeding at the beginning of each anaerobic phase could suppress the growth of
327 NOB and enrich the denitrifying PAOs (DNPAOs) ([Ge et al., 2014](#)). The nitrite
328 produced by AOB was probably denitrified immediately by denitrifying bacteria using
329 the internal carbon source in aerobic phase, which could help the enhancement of
330 phosphorus release and uptake in each sub-cycle (Fig. 3). These above-mentioned

331 results were confirmed by the subsequent analysis about microbial diversity and key
332 functional genera.

333 **3.3. Microbial population dynamics**

334 **3.3.1. Microbial diversity and richness**

335 The diversity of the microbial communities were revealed using MiSeq
336 pyrosequencing technology. The samples collected from phase I, phase II and phase
337 III were denoted G1–G3, respectively, and a total of 52,983, 45,500, and 72,341
338 sequence reads were recovered, from which 290, 226, and 256 operational taxonomic
339 units (OTUs) were identified. As shown in Table 3, the bacterial richness expressed by
340 the least Chao1 index was the lowest when the ratio of COD/N was 10. However,
341 there was little difference in the Shannon and Simpson estimators among samples. In
342 phase II, bacterial death might have caused the decrease in microbial richness, but the
343 microbial diversity was not obviously changed. It must be noted that, the plateaued
344 rarefaction curves showed that the sequenced samples were an adequate
345 representation of the population (Fig. 4A). As visualized in the Venn diagram (Fig.
346 4B), approximately 46% of the total OTUs were shared between the G1, G2, and G3
347 samples, indicating a continuous evolution of bacterial communities during the
348 granulation of PC-AGS. Therefore, these results were in conformity to previous
349 results, indicating that the richness and diversity of microbes is related to biomass
350 concentration in PC-AGS systems and that the COD/N ratio could influence the
351 bacterial richness of the microbial community.

352

353 **3.3.2. Microbial community structure**

354 The relative abundances and structures of the bacterial communities, at the phylum
355 and family levels, in the granule samples with varying COD : N : P ratios are in Fig. 6.
356 As shown in Fig. 5A, the seven phyla included Proteobacteria, Bacteroidetes,
357 Actinobacteria, Chlorobi, Firmicutes, Chloroflexi, and Spirochaetes, of which
358 Proteobacteria and Bacteroidetes were predominant in all samples, accounting for
359 88.31%, 81.27%, and 82.41% in samples G1–G3, respectively. These phyla have
360 frequently been reported in activated and granular sludges (Zhang et al., 2020),
361 implying these microbes are ubiquitous in biological wastewater treatment. Wang et al.
362 (2011) found that Proteobacteria play an crucial role in organic and nitrogen removal,
363 so they likely contributed to the PC-AGS pollutant removal performance. In addition,
364 Proteobacteria can secrete large amounts of EPS which could help the adhesion of
365 flocs to promote granulation (Liu et al., 2017). Bacteroidetes, which is a group of
366 filamentous bacteria (Du et al., 2017), has the ability to degrade refractory organic
367 compounds (Zhang et al., 2016), decreased from 12.82% (initial) to 6.93% and 8.89%
368 in phase II and phase III due to the granulation process. Furthermore, the relative
369 abundance of Chloroflexi dropped rapidly when influent conditions changed, while
370 the relative abundances of Actinobacteria, Firmicutes, and Spirochaetae increased,
371 especially if sample G1 is compared to sample G2, which implied that there was
372 strong competition among these bacteria. Actinobacteria have been found to benefit
373 sludge aggregation (Liu & Liu, 2006) and play an important nitrogen removal part in
374 wastewater treatment systems (Farghaly et al., 2017). Firmicutes are a kind of
375 carbohydrate fermenting bacteria which may also help with carbon consumption
376 (Christy et al., 2014). Lee et al. (2013) reported that Spirochaetae accelerated the
377 fermentation of amino acids and carbohydrates, making them a more consumable
378 carbon source..

379 Seventeen genera with relative abundances greater than one percent were detected
380 (Fig. 5B). Like observations at the phylum level, the genera with the highest relative
381 abundance in the different samples were also different, *Thiothrix* in G1 (36.84%),
382 *norank_f__norank_o__HOC36* in G2 (34.78%), and *Thiothrix* in G3 (34.85%). These
383 differences might have been due to the changes in influent nitrogen concentration.
384 *Thiothrix*, a filamentous bacterium that exhibits physiological activity under
385 denitrification conditions and can reduce nitrate to nitrogen (Yang et al., 2018),
386 mainly grew inside the granular structure. This can be used to explain why nitrate is
387 removed so efficiently in the PC-AGS system throughout the operation. Conventional
388 PAOs, i.e. *Candidatus_Accumulibacter*, were in the system, and were especially
389 abundant in G2 (1.97%) when influent phosphorus concentration increased. In
390 addition, some microorganisms with biological phosphorus removal abilities were
391 also found, such as *Trichococcus* and *Desulfobulbus* (Guo et al., 2016), accounting for
392 0.04% and 0.54%, 3.11% and 0.47%, and 1.83% and 1.48% in the G1–G3 samples,
393 respectively. Other dominant genera shared by G1–G3 included certain no-rank
394 genera, some of which increased (e.g. *norank_f__Chlorobiaceae* and
395 *norank_f__Propionibacteriaceae*) while others decreased (e.g.
396 *norank_f__Saprosiraceae*, *norank_f__A4b*, and *norank_f__Veillonellaceae*). It has
397 been reported that Propionibacteriaceae plays an important part in the
398 biotransformation and biodegradation of various pollutants (Song et al., 2021).
399 Chlorobiaceae has been frequently used in treating aniline wastewater, poultry
400 slaughterhouse wastewater and pharmaceutical wastewater, etc. (Ma et al., 2020).

401 **3.4.3. Key functional species**

402 The key functional genera were categorized as ammonia-oxidizing bacteria (AOB),
403 nitrite-oxidizing bacteria (NOB), denitrifying bacteria (DNB),

404 phosphorus-accumulating organisms (PAOs), denitrifying PAOs (DNPAOs),
405 sulfate-reducing bacteria (SRB), and sulfide-oxidizing bacteria (SOB) to better assess
406 the influence of microbial population dynamics on the performance of PC-AGS in
407 simultaneously removing carbon, nitrogen, and phosphorus (Table 4).

408 Biological nitrogen and phosphorus removal was related to the microorganisms in the
409 granules. Slow growing bacteria, especially PAOs, AOBs, and NOBs, have been
410 shown to promote the stability of AGS (Franca et al., 2018). Specifically, nitrogen
411 removal pathways consisted of denitrification, SND, and partial nitrification, which
412 were related to the nitrifying and denitrifying bacteria.
413 *Norank_f__Nitrosomonadaceae* were regarded as the predominant AOB, increasing
414 slightly after an initial decrease (0.01-0.03%), while *Nitrospira*,
415 *norank_f__Xanthomonadaceae*, and *norank_f__Pseudomonadaceae* were the
416 identified NOB, decreasing from 0.65% to 0.10% in relative abundance. These trends
417 indicated that the reduction in the ratio of COD/N was not conducive to the
418 enrichment of AOB and NOB. On the other hand, at least 16 DNB were identified in
419 the PC-AGS, indicating the DNB were diverse with high relative abundances and
420 richness. In addition, the relative abundance of DNB (total) reduced from 38.88% to
421 30.49% with reduction COD/N ratio, and then returned to a high level (37.82%) when
422 COD/N ratio returned to 20. The relatively high abundance of DNB in PC-AGS
423 improved the removal efficiency of TIN. Competition between PAOs and GAOs is a
424 main challenge, however, in this reactor the conventional GAOs, e.g.
425 *Candidatus_Competibacter* and *Defluviicoccus*, were not detected, while PAOs, e.g.
426 *Candidatus_Accumulibacter*, *Rhodococcus*, and *Acinetobacter*, were detected,
427 possibly because of the extended anaerobic phase. These results were in conformity to
428 He et al. (2018) who found that reduced aeration time inhibited the growth of GAOs

429 while promoting the growth of PAOs. During the operation, when influent phosphorus
430 concentration increased to 20 mg/L the abundance of PAOs peaked at 2.09%. The
431 presence of PAOs can ensure excellent $\text{PO}_4^{3-}\text{-P}$ removal efficiency even in relatively
432 low abundances (Gao et al., 2020), which is consistent with the performance of the
433 reactor. DNPAOs can be important for PC-AGS formation and nitrogen and
434 phosphorus removal (Yuan et al., 2019), and four identified DNPAOs, *Dechloromonas*
435 (0.02–0.06%), *Hyphomicrobium* (0.08–0.37%), *Aeromonas* (0.07–0.15%), and
436 *Comamonas* (0.00–0.17%), showed different tendencies in the three phases.
437 Meanwhile, a complex microbial community composed of SOB and SRB was
438 detected, especially in G3. Guo et al. (2016) found that SOB and SRB could have a
439 synergistic effect on phosphorus release and phosphorus uptake, seemingly replacing
440 the role of conventional PAOs. All these results further confirmed that mycelial pellets
441 can provide favorable conditions for key functional groups that remove pollutants and
442 promote the stability of PC-AGS.

443 **4. Conclusions**

444 In this study, a strategy to improve AGS stability and carbon, nitrogen, and
445 phosphorus removal by adding *Phanerochaete chrysosporium* was proposed and
446 tested. The results showed that PC-AGS maintained relatively high and stable carbon,
447 nitrogen, and phosphorus removal efficiencies. The stable EPS production with
448 increasing PN/PS ratios could ensure the long-term stability of PC-AGS. Moreover,
449 PC-AGS had larger granules with many channels which facilitated a large number of
450 functional microorganisms that could secrete more EPS and remove pollutants
451 efficiently. The future research should be focus on the treatment of industrial
452 wastewater containing refractory organic matter by PC-AGS.

453 **Acknowledgements**

454 The authors gratefully appreciated the financial support of Henan Provincial Science
455 and Technology Development Program (Grant No. 212102110030), and the National
456 Natural Science Foundation of China (Grant No. 21107100).

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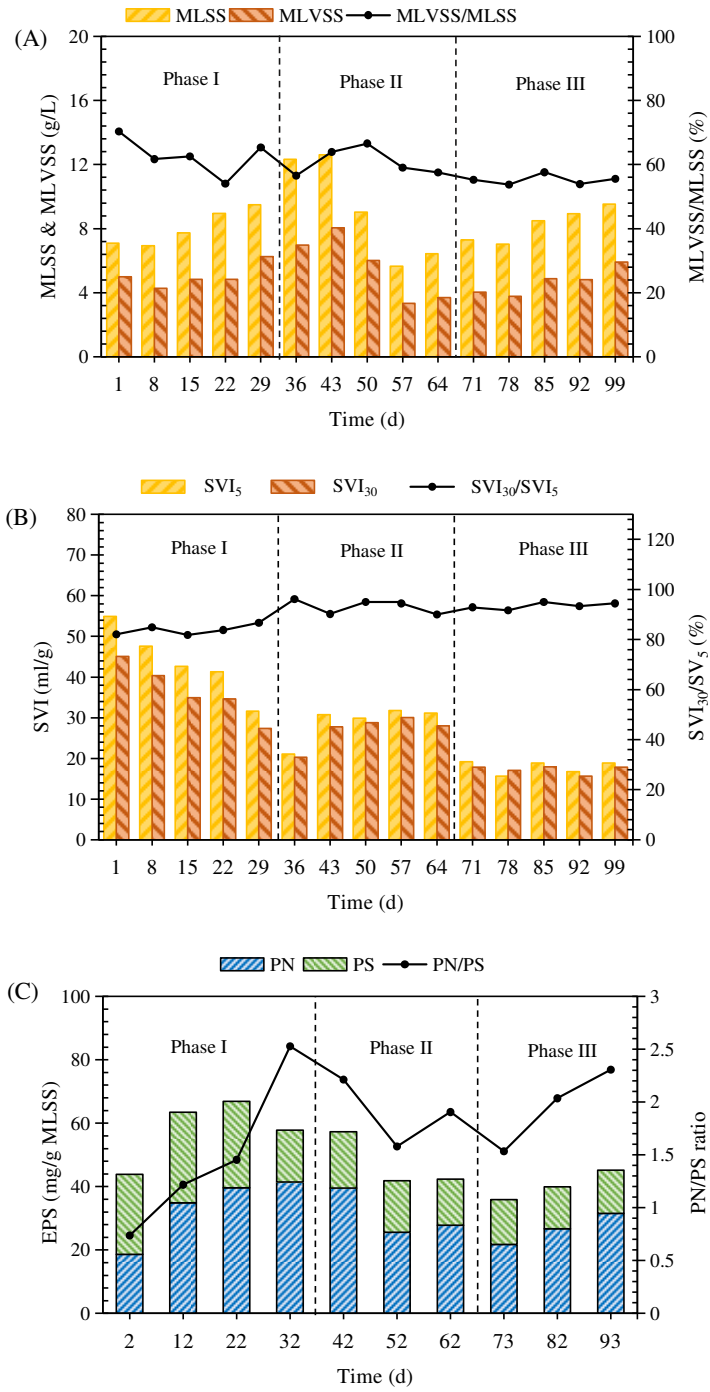


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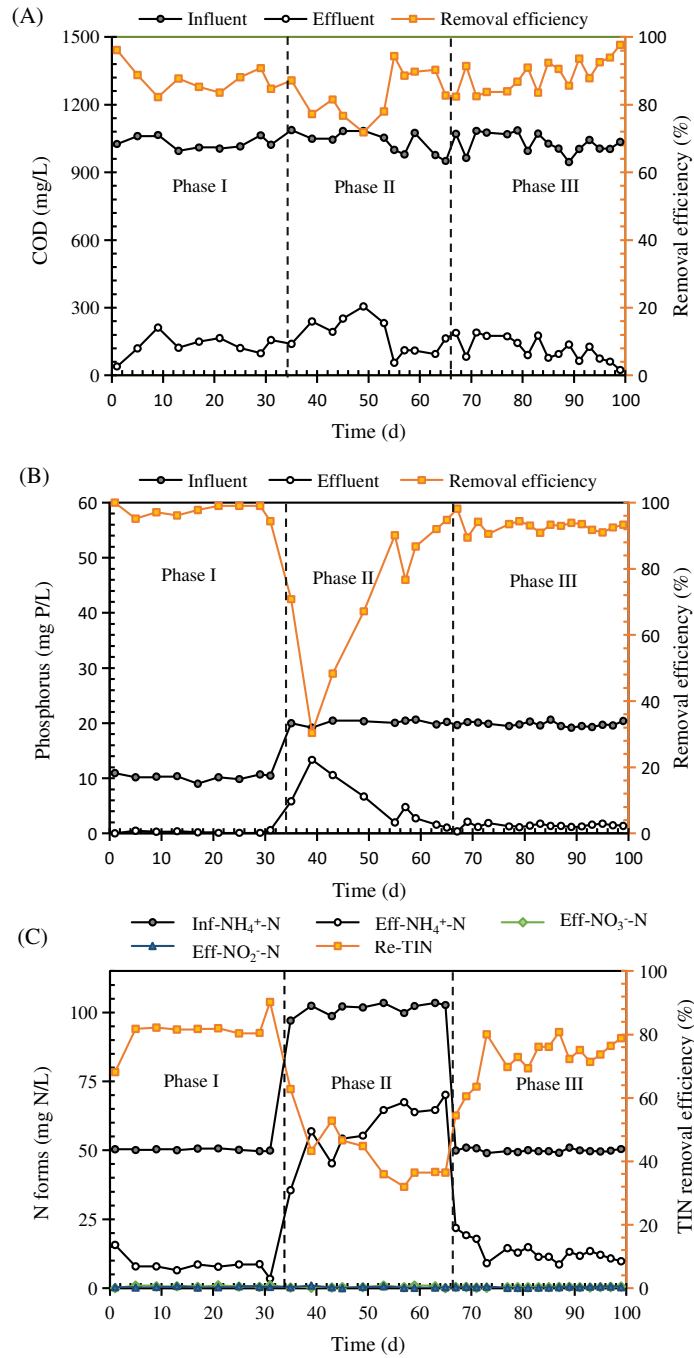


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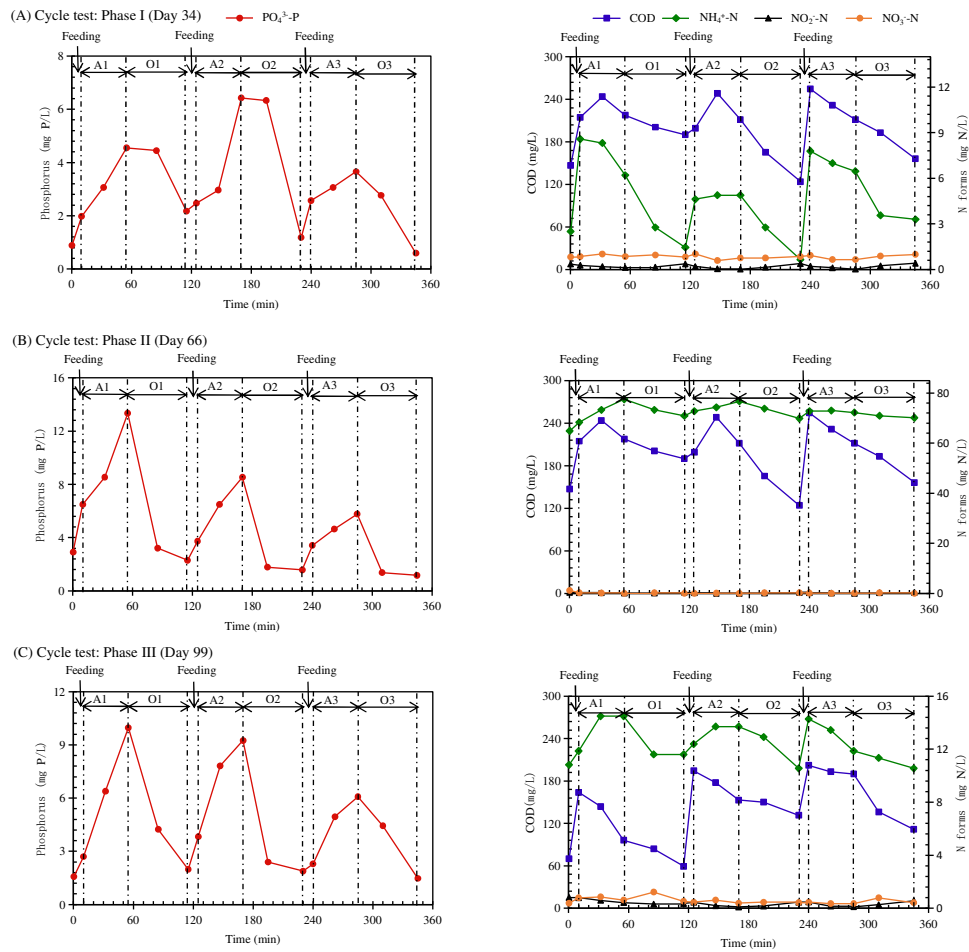


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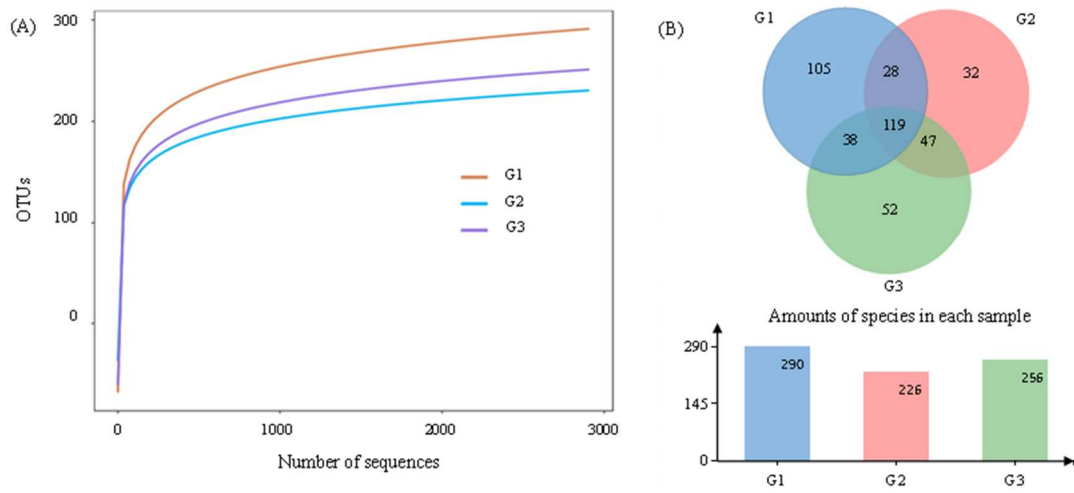


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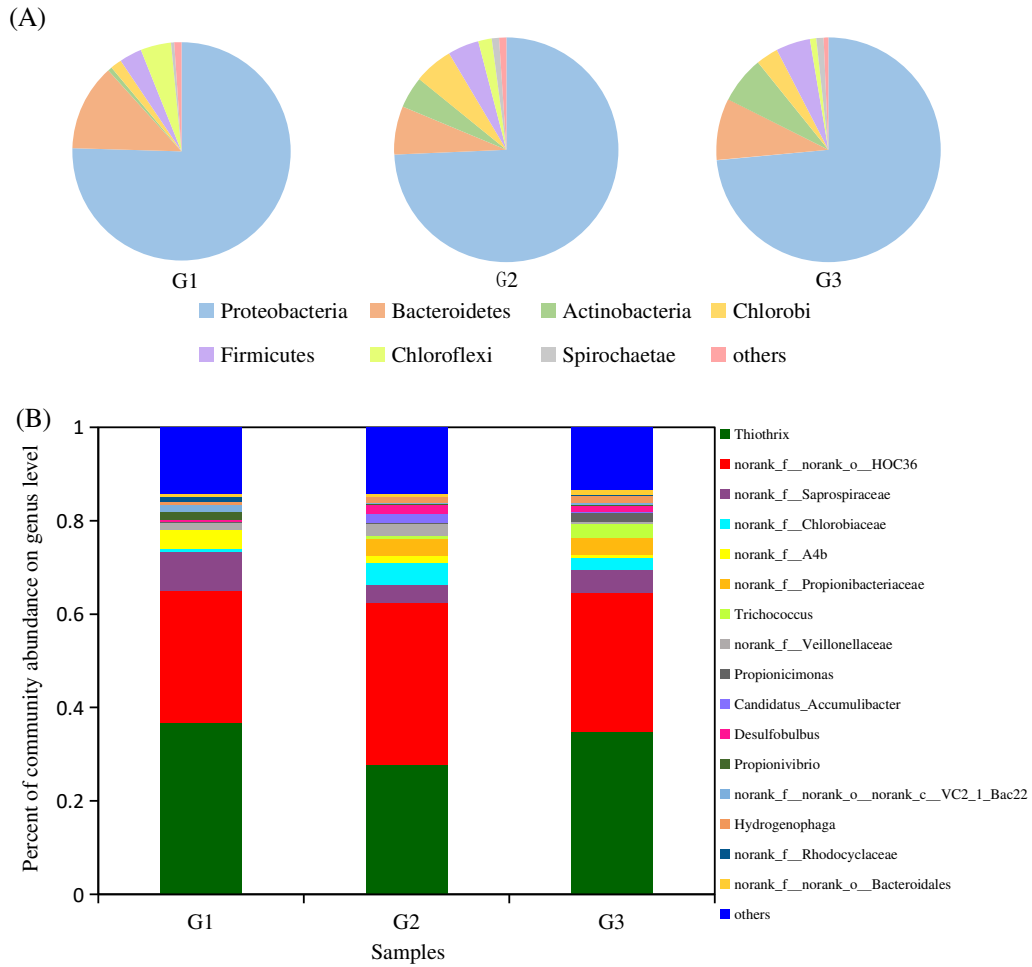


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Table 1 Composition of the synthetic wastewater.

Components	Concentration (mg/L)	Trace elements ^b	Concentration (µg/L)
Glucose	330	CoCl ₂ ·6H ₂ O	250
Potassium acetate	540	CuSO ₄ ·5H ₂ O	250
Sodium propionate	300	MnSO ₄ ·H ₂ O	250
(NH ₄) ₂ SO ₄ ^a	235, 470, 235	NiCl ₂ ·6H ₂ O	250
K ₂ HPO ₄ ^a	35, 70, 70		
KH ₂ PO ₄ ^a	15, 30, 30		
CaCl ₂	23		
MgSO ₄ ·7H ₂ O	22		
FeSO ₄ ·7H ₂ O	9		
NaHCO ₃	100		

^a These values were regularly increased according to the three periods (phase I, II, III)

^b The micronutrient solution added in the synthetic wastewater were used for microbial growth.

Table 2 Feeding conditions during the whole experiment.

Phase	Time (day)	Organic loading rate (kg COD/L/d)	Nitrogen loading rate (mg N/L/d)	Phosphorus loading rate (mg P/L/d)	Ratio (COD: N: P)
Phase I	1–34	2.91 ± 0.10	146.54 ± 9.45	28.62 ± 1.46	100 : 5 : 1
Phase II	35–66	2.90 ± 0.13	288.97 ± 9.96	57.55 ± 2.26	100 : 10 : 2
Phase III	67–100	2.89 ± 0.12	138.37 ± 3.11	55.06 ± 1.51	100 : 5 : 2

Table 3 Species richness and diversity of G1, G2, and G3.

Samples	Effective reads	Observed OTUs	Community richness		Community diversity	
			Chao1 ^a	Shannon ^b	Simpson ^c	
G1	52,983	290	290	3.6663	0.78027	
G2	45,500	226	226	3.8521	0.80049	
G3	72,341	256	256.1	3.7781	0.79704	

^aA larger number → greater richness.

^bA larger number → more diversity.

^cA larger number → less diversity.

Table 4 Relative abundances of the key functional groups classification at genus level.

Key functional groups		Relative abundances (%)					
		G1		G2		G3	
AOB	<i>norank_f__Nitrosomonadaceae</i>	0.03		0.00		0.01	
	<i>Nitrospira</i>	0.00		0.01		0.00	
NOB	<i>norank_f__Xanthomonadaceae</i>	0.57	0.65	0.55	0.56	0.10	0.10
	<i>norank_f__Pseudomonadaceae</i>	0.08		0.00		0.00	
	<i>Thiothrix</i>	36.84		27.69		34.85	
	<i>Zoogloea</i>	0.01		0.19		0.12	
	<i>Thauera</i>	0.02		0.16		0.27	
	<i>Rhodobacter</i>	0.31		0.65		0.75	
	<i>Chryseobacterium</i>	0.06		0.00		0.01	
	<i>Hydrogenophaga</i>	0.63		1.23		1.44	
	<i>Devosia</i>	0.04		0.02		0.01	
DNB	<i>norank_f__Rhodospirillaceae</i>	0.05	38.88	0.02	30.49	0.00	37.82
	<i>norank_f__Comamonadaceae</i>	0.49		0.27		0.17	
	<i>Rubrivivax</i>	0.06		0.00		0.00	
	<i>Paracoccus</i>	0.00		0.09		0.07	
	<i>Mesorhizobium</i>	0.00		0.00		0.01	
	<i>Acidovorax</i>	0.09		0.09		0.03	
	<i>Desulfovibrio</i>	0.00		0.07		0.08	
	<i>Pseudoxanthomonas</i>	0.03		0.00		0.01	
	<i>Flavobacterium</i>	0.25		0.01		0.00	
	<i>Candidatus_Accumulibacter</i>	0.02		1.98		0.04	
PAOs	<i>Rhodococcus</i>	0.00	0.03	0.00	2.09	0.01	0.11
	<i>Acinetobacter</i>	0.01		0.11		0.06	
	<i>Dechloromonas</i>	0.03		0.06		0.02	
DNPAOs	<i>Aeromonas</i>	0.15	0.43	0.07	0.56	0.07	0.43
	<i>Hyphomicrobium</i>	0.08		0.37		0.34	
	<i>Comamonas</i>	0.17		0.06		0.00	
SRB	<i>Desulfomicrobium</i>	0.02	0.49	0.02	1.85	0.00	1.48
	<i>Desulfobulbus</i>	0.47		1.83		1.48	
SOB	<i>Thiobacillus</i>	0.03	0.07	0.00	0.54	0.02	3.13
	<i>Trichococcus</i>	0.04		0.54		3.11	