Fast development of purple nonsulfur bacteria (PNSB) in a bubble column photobioreactor: influence of carbon source and dissolved O_2 availability on wastewater treatment performance

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Abstract

Photobioreactors with purple nonsulfur bacteria (PNSB) constitute a promising technology for wastewater treatment, gas purification, and high value-added bioproducts. In this work, different operational strategies were tested to investigate their impacts on photobioreactor performance, biomass properties, and microbial community. It showed that the operational conditions significantly influence the performance and microbial composition of PNSB photobioreactors. Anaerobic conditions favored the dominance of *Rhodopseudomonas* spp. and moderate COD removal efficiency (36.7%), while aerobic conditions significantly enhanced COD removal efficiency (91.7%) but altered the microbial community composition from PNSB to aerobic heterotrophic microorganisms, such as *Delftia sp.* and *Microbacterium sp.*. The removal ratio of chemical oxygen demand (COD):N:P was 100:10:2 under anaerobic conditions with continuous CO_2 supply, while it was 100:1:1 under aerobic conditions with continuous supply of a CO_2/O_2 mixture. Net CO_2 removal efficiencies of 2.8% and 5.4% were recorded under anaerobic conditions with and without the addition of organic carbon, respectively. The outlet CO_2 concentration showed that CO_2 uptake occurred under dark and anaerobic conditions without organic carbon. The reduced production of extracellular polymeric substances (EPS) was observed after COD was removed from the influent, preventing granule formation.

Keywords: Anaerobic condition, CO_2 uptake, COD removal, Microbial community, Wastewater treatment.

1. Introduction

The global rise in wastewater production and the environmental challenges associated with its management require sustainable and effective treatment methods. Traditional physio-chemical treatment processes, although effective, often come with high operational costs, chemical usage, and secondary pollution [1, 2]. Purple nonsulfur bacteria (PNSB) offer a biological alternative that could address some of these challenges, due to their versatile metabolic capabilities and ability to thrive in various environmental conditions [3].

PNSB have been applied in photobioreactors for wastewater treatment and gas purifying by their ability to thrive in anaerobic conditions and utilize a wide range of carbon sources and nutrients, which will be converted into bioenergy and useful bioresources [4-6]. A complex interaction of metabolisms can be deployed by PNSB, including photoheterotroph, photoautotroph, respiration, fermentation, and nitrogen fixation, depending on light/dark conditions, carbon source, and O_2 availability [7]. In aerobic conditions, PNSB can grow by respiration, while in anaerobic conditions with light, they grow as photoautotrophs with CO_2 as the sole carbon source or grow as photoheterotrophs with organic carbon sources, such as organic acids, sugars, and volatile fatty acids. Various configurations of PNSB photobioreactors were applied and evaluated, including batch reactor and continuous column reactor [6], to treat municipal wastewater, soybean wastewater, agricultural wastewater, etc. Most of PNSB wastewater treatment systems were reported with a strong ability on chemical oxygen demand (COD) removal, with over 70% removal efficiency [8, 9]. Previous research showed that in anaerobic membrane bioreactors (AnMBR) treating domestic wastewater, total N and total P removal efficiencies could reach up to 92% and 98%, respectively [10].

However, in natural environments, PNSB always coexist with various microorganisms. Compared with other bacteria, PNSB have a lower growth rate [11]. The slow growth rate makes it challenging for PNSB to outcompete with other microorganisms for nutrients and spaces, preventing its enrichment from the initial inoculum containing a mixed microbial community. PNSB can assimilate carbon (C), nitrogen (N), and phosphorus (P) from wastewater with the COD:N:P ratio of 100:6.4:1.1 [12]. Due to the complicated factors and requirements, the bubble column photobioreactor is a promising system for the efficient cultivation of PNSB due to its high mass transfer rate and, uniform mixing, and adjustable light and gas availability [13].

It was reported that *Rhodobacter* could be enriched under high acetate concentrations (5-20 mmol/L), while *Rhodopseudomonas* was enriched at lower concentrations (0.5-1 mmol/L) [14]. The optimum temperature of PNSB was 25 °C-30 °C [15]. The pH of 7-8 was preferred for PNSB growth. The highest organic carbon removal and lowest inorganic carbon removal were observed at pH 8.0, while pH 5.0 showed the highest inorganic carbon removal [16]. In addition, the highest maximum specfic growth rate of *Rhodopseudomonas palustris* was achieved at a light intensity of around 1800 μmol/m^2/s [17]. PNSB granules were successfully enriched out of activated sludge in a sequencing batch reactor (SBR) with acetate-based synthetic water and achieved promising COD and nutrient removal efficiencies of 95% and over 70%, respectively [18]. However, it took five months to inoculate the biomass in batch mode before switching to SBR mode. Most other studies focusing on reactor performance, bioproducts, or growth kinetics of PNSB, used enriched PNSB culture instead of mixed inoculum, which was easier to gain [19, 20]. Therefore, methods for the fast enrichment of PNSB to promote the development of PNSB

applications are needed.

Moreover, PNSB granules are desired to facilitate biomass harvesting due to their enhanced settling ability. Biomass granulation can also improve the reactor performance by increasing the solid retention time (SRT), resistance to environmental stresses, and microbial activity. Aeration plays a vital role in the biomass granulation process to maintain the microbial structure and the metabolism pathways. Prior research showed that high air velocity can provide shear force to stimulate the production of extracellular polymeric substances (EPS), which are critical for the formation and maintenance of granules [21, 22]. In contrast, *Rb. capsulatus* might produce a heat-labile metabolites inducing disaggregation. Higher COD/N ratios could also lead to disaggregation because of the overgrowth of filamentous bacteria [23].

This study aimed to approach strategies for the fast development of PNSB in a simple bioreactor configuration. For this purpose, a bubble column photobioreactor that was continuously supplied with different ratios of CO_2 and O_2 was implemented. The reactor was inoculated with activated sludge due to its wide availability in bulk amounts. Different operational strategies were applied to investigate their impacts on the reactor performance (COD, nitrogen and phosphorous removal), biomass properties (chlorophyll, carotenoids, and exopolysaccharide content), and the resulting microbial community.

2. Materials and methods

2.1. Synthetic wastewater and inoculum

Medium-strength synthetic wastewater with a COD of 475.5±10.4 mg/L modified according to García et al. was fed to the reactor [24]. $NaHCO_3$ and $CaCl_2$ at

3 and 0.005 g/L, respectively were added to keep the pH above 7 and to promote biomass granulation. The composition of the medium-strength synthetic wastewater was (g/L): CH_3COONa (0.6), NH_4Cl (0.31), K_2HPO_4 (0.073), $NaHCO_3$ (3), and $CaCl_2$ (0.005). In addition, 10 mL trace element stock solution was added to 1 L of media with the following composition (g/L): EDTA-$C_{10}H_{16}N_2O_8$ (0.5), $FeSO_4 \cdot 7H_2O$ (0.2), $ZnSO_4 \cdot 7H_2O$ (0.01), $MnCl_2 \cdot 4H_2O$ (0.003), H_3BO_3 (0.03), $CoCl_2$ (0.011), $CuCl_2 \cdot 2H_2O$ (0.162), $NiCl_2 \cdot 6H_2O$ (0.002), $NaMoO_4 \cdot 2H_2O$ (0.003), and $MgSO_4 \cdot 7H_2O$ (0.02). Secondary activated sludge from a wastewater treatment plant performing nitrification-denitrification was used as the inoculum (Querétaro, Mexico). The reactor was inoculated with 10% (v/v) activated sludge relative to the working volume of the reactor (3.6 L), reaching an initial total suspended solids (TSS) concentration of 0.32 g TSS/L.

2.2. Reactor setup and operation

As shown in Figure 1, a laboratory-scale cylindrical column (62.5 cm height, 8.7 cm inner diameter, total volume of 3.7 L) was operated at a working volume of 3.6 L. Magnetic stirring (150 rpm) was provided at the bottom to keep the biomass suspension. The reactor was operated with 12/12 h light/dark photoperiods at room temperature (Figure. 1). An average light intensity of ~200 μmol/m^2/s was provided by a dual strip LED light (Model MNSL, Lithonia Lighting, Mexico), which was measured inside the reactor at the bottom, middle and top of the column. The 12/12 h photoperiod was controlled by a digital control unit connected to a Lab-Quest data acquisition card (Venier, Beaverton, OR, USA). To control the concentration of CO_2 flowing into the reactor, CO_2 and air or O_2 were supplied at desired flow rates via mass flow controllers (Model GFC, Aalborg Instruments, New York, USA) and mixed in a gas chamber before entering the column. The mixed gas was introduced to

the bottom of the column by a stone diffuser at a total flow rate of 0.2 L/min. A settler of 0.9 L was implemented to avoid biomass washing out in the column. Since the CO_2 supply could lead to acidification of the culture medium, which was detrimental to the cultivated organisms, 3 g/L of $NaHCO_3$ was added to the influent to keep the pH above 7. The column was equipped with dissolved oxygen (DO), pH, and temperature sensors, as well as a gas sensor to monitor the CO_2 concentration at the gas outlet. DO, pH, temperature and CO_2 concentration data were acquired every hour throughout the experiment.

The liquid phase of the photobioreactor was operated in batch mode for 11 days, while the gas phase was supplied continuously at a gas retention time (GRT) of 18 min, determined as follows:

$$GRT = V/F \quad (1)$$

where V and F stand for the working volume of the reactor and the gas flow rate, respectively. Thereafter, the reactor was also operated in continuous mode. For this purpose, the liquid influent, effluent, and biomass recirculation were controlled by three automated peristaltic pumps (model 77200-50, Cole-Parmer, USA), with flow rates of 1, 2, and 1 mL/min, respectively, resulting in a hydraulic retention time (HRT) of 2.5 days. Table 1 summarizes the COD concentrations in wastewater and gas compositions tested. In phase I, II, and III, a mixture of CO_2 and N_2 was supplied to keep the reactor in anaerobic condition, while in phase IV, a mixture of CO_2 and air was supplied to keep the reactor in aerobic condition. A DO sensor was used to monitor the anaerobic/aerobic condition throughout the experiment. In phase III, COD in the form of acetate was not supplied to evaluate the impact of carbon source on the metabolism pathways of the microorganisms.

2.3. Molecular biology analysis

Mixed liquor samples (50 mL) from the reactor were periodically drawn on days 0, 28, 41, and 57, and stored at −20 °C for microbial analysis. Genomic DNA was extracted with PowerSoil® DNA isolation kit (MOBIO, USA). The extracted DNA samples were submitted to the Research and Testing Laboratory (RTL, Lubbock, USA) for Illumina MiSeq sequencing analysis of microalgal (18S rRNA, primers: EukA7F 5′-AACCTGGTTGATCCTGCCAGT-3′, EUK555R 5′-GCTGCTGGCACCAGACT-3′) and bacterial cells (16S rRNA, primers: 28F 5′-GAGTTTGATCNTGGCTCAG-3′, 388R 5′-TGCTGCCTCCCGTAGGAGT-3′) [25]. The resultant sequences were processed utilizing DADA2 v1.26 within the R environment. Forward and reverse reads were filtered and truncated to 200 and 250 nucleotides, respectively, chimera sequences were removed, and an amplified sequence variant table was obtained. Taxonomic classifications were assigned to representative sequences through the application of a Naïve Bayesian classifier, and two different dataset references, SILVA 132 and PR2 5.0, were used for 16S and 18S, respectively [26].

2.4. Analytical methods

The liquid samples were filtered through 0.22 μm nylon membranes before the chemical analysis. The soluble COD and NH_4^+ concentrations were determined by USEPA reactor digestion method (Hach 8000) and salicylate method (Hach 10031) respectively. Concentrations of NO_2^-, NO_3^-, and PO_4^{3-} were measured by ion chromatography (IC) system (model ICS 1500, Dionex, Sunnyvale, CA, USA). TSS and volatile suspended solids (VSS) were determined according to Standard Methods [27].

Chlorophyll and carotenoids were determined according to Osório et al. with 100% methanol [28]. Briefly, 10 mL mixed liquor was centrifuged at 2500 rpm for 10

188 min. The supernatant was discarded and 100% methanol was added to the final
189 volume of 10 mL. The mixture was resuspended by a vortex. For sufficient extraction,
190 the mixture was kept at 4 ℃ in dark for over 12 h. Then, the mixture was centrifuged
191 at 2500 rpm for 10 min to obtain a clear supernatant. The absorbance of the
192 supernatant was measured in a 1-cm cuvette at the wavelengths of 480 nm, 632 nm,
193 652 nm, 665 nm, 696 nm, and 750 nm in duplicate by a spectrophotometer (model
194 VE-5600UV, Velab). The calculations of chlorophyll a, chlorophyll b, chlorophyll c,
195 chlorophyll d, and carotenoids concentrations can be found in Osório et al 2020 [28].
196 EPS was extracted by a modified heating method [29]. Briefly, 10mL mixed
197 liquor sample was centrifuged at 5000*g* for 15 min. The supernatant was filtered
198 through 0.22 μm nylon membrane, and the filtrate was collected to represent the
199 loosely bound EPS (LB-EPS) fraction. The supernatant was discarded, and the
200 residual biomass was resuspended with 0.9% (w/v) NaCl solution to the original
201 volume (10 mL). The mixture was heated at 70 °C for 30 min. The extracted solution
202 was centrifuged at 10,000*g* for 20 min under 4 °C, and the supernatant was filtered
203 through 0.22 μm nylon membrane. The filtrate was collected to represent the tightly
204 bound EPS (TB-EPS) fraction. The EPS samples are stored at −20 °C prior to
205 analysis. Extracellular polysaccharides (PS) and proteins (PN) are measured by the
206 colorimetric method and the Lowry-Folin method, respectively [30, 31].
207
208 **3. Results and discussion**
209 **3.1. COD and nutrients removal performance**
210 COD removal performance is shown in Figure 2a. During batch operation in
211 the experimental phase I, with continuous CO_2 and N_2 supply, the COD concentration
212 was reduced from 551.5 mg/L to 353.5 mg/L within 11 days (corresponding to a
213 removal efficiency of 35.9%). During phase II, under HRT of 2.5 days and continuous

supply of CO_2 and N_2 gas, the COD removal was stable with an average removal efficiency of 36.7%, corresponding to an average removal rate of 68.8 g COD/m^3·d (Table 2). In the experimental phase II, CO_2 and N_2 were continuously supplied, which stripped the O_2 potentially produced via photosynthesis. Since no DO was available (Figure. 2C), COD removal was attributed to photoheterotrophic metabolism during the light period and respiration during dark period (Figure 3). A clear increase of biomass concentration (TSS) in the photobioreactor was observed, passing from 0.5 g/L at the end of experimental phase I to 0.7 mg/L at the end of experimental phase II. An average biomass specific growth rate of 0.10/day was recorded under the experimental conditions set in experimental phase II (Table 2).

Previous research showed that when using purple phototrophic bioreactors to treat raw domestic wastewater in membrane systems, above 90% COD removal was achieved [12]. In those cases, the soluble COD concentrations of the influent were 245 mg/L and 138 mg/L, respectively, which were much lower than that of this research. A recent study using purple phototropic bacteria in an anaerobic membrane bioreactor (PAnMBR) for refinery water treatment achieved a maximum soluble COD removal of 75% [32]. COD/Biomass ratio in this study was 0.8 g COD/g VSS, which was lower than most prior studies (1-4 g COD/g VSS) which showed over 50% removal of COD with HRT of 1.5-4 days [4, 33, 34]. The COD:N:P removal ratio in this study was consistent with previous research on PNSB (will be discussed later in this section), indicating that the main metabolisms during the process were acted by PNSB in this study. However, in other studies, the bioreactors were operated longer than this study, which ensured that the biomass adjusted well to the environment. Note that the reactor in this study was seeded with activated sludge. The shift of microbial community resulting from different operational conditions can cause

239 different COD removal performances.

240 In order to compare the impact of carbon source on the reactor performance,
241 organic carbon source was eliminated during the experimental phase III, and the COD
242 concentration was progressively decreased from 223 mg/L to 42.5 mg/L in the
243 effluent. The higher COD values recorded in the first days of experimental phase III
244 were probably attributed to the biomass decay and the carryover of COD from phase
245 II (Figure 2b). Sudden changes in COD have may trigger biomass decay in
246 photobioreactors [24]. The SRT of the system was 6.4 days. It is expected to take
247 around 10 days to decay more than half of biomass.

248 During phase IV, a mixture of air and CO_2 was fed to the reactor continuously,
249 while organic carbon was reinstated in the influent. A COD removal efficiency of up
250 to 91.7% was achieved in phase IV due to the presence of O_2 in the system. This
251 oxygen promoted the proliferation of arobic heterotrophic microorganisms that use
252 organic matter as carbon and energy sources, and employ more efficient metabolism
253 pathways to break down organic compounds. This results in a higher COD removal
254 efficiency compared to anaerobic conditions. An average removal rate of 175.8 g
255 COD/m^3·d and an average specific COD removal rate of 311.8 g COD/kg VSS·d
256 indicated that the reactor reached a high COD removal performance with the presence
257 of O_2. The presence of O_2 promoted the respiration of heterotrophs, which used COD
258 as the carbon source. In the algal photobioreactor with intermittent illumination, COD
259 removal could reach around 70% [35], which was lower than the photobioreactor in
260 this study. As it will be discussed in detail in Section 3.5, changes in COD and O_2
261 availability indeed produced profound changes in the predominant microbial
262 communities in each experimental phase tested.

263 The experimental conditions in experimental phases II and IV allowed for

264 COD removal in the photobioreactor with the best performance observed in phase IV
265 due to O_2 availability, which promoted the heterotrophic metabolism. If a CO_2-rich
266 gas stream is intended for use as an inorganic carbon source in photobioreactors for
267 wastewater treatment, it is recommended to mix this stream with air to ensure an
268 adequate supply of O_2 and facilitate high COD removal. Additionally, the conditions
269 established during experimental phase II also accomplished the removal of organic
270 matter at a rate of 68.8 $g/m^3 \cdot d$. Moreover, switching to aerobic conditions (as in phase
271 IV) for a brief period can be used as a polishing strategy for COD removal if required.
272 As for the nutrient removal, NH_4^+-N was reduced from 72.3 mg/L to 43.6
273 mg/L, and PO_4^{3-}-P was reduced from 20.9 mg/L to 11.5 mg/L for the batch operation
274 of the reactor with during phase I (Figure 3). The removal ratio of COD:N:P was
275 100:15:5. During phase II, the removal efficiencies of NH_4^+-N and PO_4^{3-}-P were
276 26.6% and 11.9%, respectively. The removal ratio of COD:N:P was 100:10:2, which
277 was consistent with previous research on purple bacteria in photo anaerobic
278 membrane bioreactor (100:6.4:1.1) [12]. Since the uptake of PO_4^{3-}-P by purple
279 bacteria under anaerobic condition was lower than that under aerobic condition and
280 the uptake of PO_4^{3-}-P by phosphate-accumulating organisms (PAOs) required both
281 anaerobic and aerobic conditions. While only anaerobic condition was provided
282 during phase II, a limited PO_4^{3-}-P removal efficiency was observed.
283 During phase III, the average NH_4^+-N removal performance was similar to
284 phase II. The PO_4^{3-}-P average removal efficiency improved to 27.1% due to the
285 growth of biomass (VSS) compared with phase II, since the average specific removal
286 rate did not improve.
287 During phase IV, the NH_4^+-N removal decreased, with an average removal
288 efficiency of 10.0%. The removal ratio of COD:N:P was 100:1:1. The PO_4^{3-}-P

289 removal performance increased because O_2 in the gas inlet provided an aerobic
290 condition, and anaerobic condition was provided inside the biomass granular. So that
291 the two processes (P release and P accumulation) for PO_4^{3-}-P removal can be
292 completed.

293 Previous research showed that in a continuous PNSB photobioreactor, NH_4^+-N
294 and PO_4^{3-}-P removal rates reached up to 113 g N/m^3·d and 15 g P/m^3·d, respectively
295 [18]. In other studies with PNSB wasterwater treatment, the removal efficiencies of
296 NH_4^+-N and PO_4^{3-}-P were more than 60% and 55%, respectively [8, 34, 36]. These
297 were much higher than the results in this study. However, the previous research
298 applied a higher SRT (11 days) and a higher biomass concentration of up to 4 g
299 VSS/L, over 4 times more than this study [18].

300 **3.2. CO_2 uptake and the impact of carbon source**

301 The average CO_2 removal efficiency was 2.8% in phase II (Figure 4a). The
302 average removal efficiency of CO_2 increased to 5.4% in phase III. The CO_2 removal
303 rates in phases II and III were 0.11g/(L day) and 0.22 g /(L day), respectively. CO_2
304 was the only carbon source of the reactor in phase III, and purple bacteria could
305 utilize CO_2 for photosynthesis when there was no other carbon source or oxygen.
306 During phase IV, there was almost no CO_2 removal from the system, due to the
307 aerobic respiration of bacteria with the presence of organic carbon. O_2 and organic
308 carbon were used by biomass in the reactor, including purple bacteria for aerobic
309 respiration and CO_2 was generated as a byproduct. In general, the conditions tested in
310 this study did not support effective CO_2 removal in the photobioreactor. However, the
311 outlet CO_2 concentration had a periodical change within each operation cycle (1 day),
312 where there were obvious peak in the light phase and valley values in the dark phase
313 during each day (Figure. 4b). It meant negativa removal of CO_2 with light and

positive removal of CO_2 in dark. Therefore, for practical applications, a potential strategy is to intermittently introduce CO_2 during the dark phase to prevent negative removal during the light phase. This approach optimizes the gas purification performance, carbon utilization, and energy costs associated with the gas supply for the system.

Under anaerobic conditions with light, purple bacteria can utilize CO_2 as a carbon source for photosynthesis in the absence of organic carbon. During phase III, with CO_2 being the sole carbon source for photosynthesis, PNSB was able to consume more CO_2. This led to an improvement in CO_2 removal efficiency, which was nearly double that of phase II. Moreover, when both organic carbon and CO_2 are present, the bacteria prioritize using organic carbon as their primary carbon source. However, in the presence of O_2, purple bacteria will undergo aerobic respiration rather than photosynthesis, consuming organic carbon instead of CO_2.

Meanwhile, in aerobic conditions, heterotrophic bacteria will also perform respiration and produce CO_2. Therefore, CO_2 removal efficiency in photobioreactors depends on their consumption by photosynthesis and their production by respiration. In an anaerobic/aerobic photobioreactor treating textile wastewater, a negative removal efficiency of inorganic carbon was reported for the entire experimental period due to the CO_2 produced from the anaerobic degradation of the organic matter. The CO_2 produced was consumed in the following aerobic photobioreactor by algae [37], while positive CO_2 production was observed during the anaerobic phase. It showed that the highest inorganic carbon removal efficiency was observed at pH 5.0, while the lowest removal efficiency was found at pH 8.0 because of the bicarbonate degradation at the lower pH [16]. This might be one of the reasons for the low CO_2 removal in this study. The CO_2 fixation rate was reported in the range of 0.1-1.5

g/Lday in photobioreactors with different illumination durations each day [38-40]. A prior study demonstrated that CO_2 removal rate by PNSB was 0.56 g/(L· day) with 12h-12h light-dark cycle [38], which is significantly higher than the rates observed in this study. However, the biomass concentration in that study was 2 g/L, considerably higher than this study (< 1 g/L). The lower biomass concentration in the reactor resulted in low CO_2 removal efficiency. Moreover, their calculations of CO_2 fixation rate were based on the production of biomass instead of the CO_2 removal in gas phase, and organic carbon uptake was not counted. This study presented a direct measurement of CO_2 removal based on the change of CO_2 concentration in gas phase.

3.3. Biomass properties

It showed that the biomass had a high chlorophyll b content and a small carotenoid content, which are the main pigments of purple bacteria (Figure. 5a). For some algal species, the content of chlorophyll a is higher than chlorophyll b (which would be explained in Section 3.5) [41], so the low chlorophyll a content indicated that algae was not dominant in the reactor. Since the values of cholorophyll a, c, and d were close to 0, the total chlorophyll was mainly contributed by chlorophyll b. The total chlorophyll content increased from 2 mg/g VSS to 14 mg/g VSS during phase II and phase III due to the benefit of anaerobic conditions for purple bacteria. It decreased significantly to the initial value (2 mg/g VSS) in phase IV because the aerobic condition promoted the growth of other bacteria. The pigment contents revealed the switch of microbial community composition, indicating that anaerobic conditions could promote the growth of purple bacteria by reducing competition with other microorganisms.

Prior research has shown that EPS played an important role in the formation and maintenance of granules derived from activated sludge granular (AGS). EPS can

increase the hydrophobicity of the cells' surface, and increasing the ratio of protein to polysaccharide can reduce the negative surface charge of cells, resulting in the decrease of their electrostatic repulsion. The changes of hydrophobicity and surface charge can promote the granulation process [42]. EPS could also protect biomass from harsh conditions such as extreme temperatures, pH fluctuations, and toxic substances [43]. However, high EPS production may reduce microbial growth rates, as more energy is expended in synthesizing EPS rather than biomass [44]. Excessive EPS can also cause clogging in the bioreactor, hinder mass transfer, and increase operational costs due to more frequent cleaning and maintenance requirements [45]. The initial sludge had the highest EPS content (Figure 5b). It decreased rapidly because of limited COD in batch mode throughout the time. In phase II, the biomass had a higher EPS content during the first week (day 18) and decreased later. The EPS content remained consistent with the end of phase II throughout phase III. In contrast, the chlorophyll content peaked during this phase, indicating the highest distribution of purple bacteria in the biomass. With organic carbon and O_2 provided in phase IV, EPS production and PN/PS ratio increased, indicating an appropriate granular formation condition. The initial activated sludge and the biomass in phase IV had higher EPS contents, consistent with that aerobic condition and organic carbon can promote EPS production. However, during the experiment, no mature granules were observed. The reactor was operated under a continuous flow mode, lacking settling time and selection pressure that promotes granule formation. In phase III, the limited organic carbon source led to a decrease in EPS production, which prevented the further granulation process. Besides, the lack of filamentous microorganisms might be another reason for the lack of mature granules. Although the system produced more EPS than conventional activated sludge system [46, 47], indicating the potential for

granule formation, it still produced much less EPS than other PNSB granule systems (over 200 g EPS/g VSS) operated more than 200 days [48]. The short duration of anaerobic phase with organic carbon in this study might be another factor causing the low EPS production and lack of granular formation.

The changes in COD and O_2 availability induced changes in the microbial communities, which could be observed via drastic color changes in each experimental phase (Figure. 6). In phase II, the color changed from yellow to brown, kept in brown during the whole phase III, and became much lighter at the end of phase IV, indicating that purple bacteria were the dominant taxa in phases II and III and decayed in phase IV, which can be confirmed by microbial analysis in section 3.5.

3.4. Microbial community characterization

16S rRNA analysis indicated that there was almost no overlap between the dominant species of sludge used as inoculum and in the later phases (Figure 7a). The most dominant species in the sludge inoculum were *Candidatus Competibacter sp.* and *Caldilinea sp.*, with relative abundances of 9.2% and 9.0%, respectively. In phase II and phase III in anaerobic conditions, *Rhodopseudomonas* spp., a PNSB, became the dominant species with relative abundances of 92.8% and 94.6%, respectively. The relative abundances of the second dominant species, *Thiobaca sp.,* which is a purple sulfur bacterium (PSB), were 2.6% and 1.2%, respectively. The relative abundances of *Thiobaca sp.* were much lower than *Rhodopseudomonas* spp. since there was limited sulfur source. In phase IV with aerobic condition, the relative abundance of *Rhodopseudomonas* spp. decreased to 36.6%. *Delftia sp.* and *Microbacterium sp.* became the other two dominant species with relative abundances of 18.5% and 13.6%, respectively, which contributed to the organic matter removal and nitrogen removal [49, 50]. The aerobic condition did not help the recovery of microorganisms to the

414 initial composition but helped develop some aerobic bacteria species. 18S rRNA
415 analysis showed the relative abundances of algae species (Figure 7b). It showed
416 almost no algae in the sludge used as inoculum (0.13%). In phase II and phase III,
417 algae became the dominant Eukaryote. The relative abundances of *Chlorella*
418 *sorokiniana,* which was the main algae species, were 84.5% and 75.6%, respectively,
419 while it dropped to 6.2% in phase IV. The main pigment of *Chlorella sorokiniana* was
420 chlorophyll a [51]. As it was mentioned in Section 3.4, there was almost no
421 chlorophyll a content in the biomass. Thus, PNSB was the main dominant taxa in the
422 reactor instead of algae.
423 *Rhodopseudomonas* spp. could perform photosynthesis under anaerobic
424 conditions with light and various carbon sources, while most other microorganisms
425 require O_2 and organic carbon to grow, which allowed *Rhodopseudomonas* spp. to
426 outcompete with other microorganisms and became the dominant species. Since
427 *Rhodopseudomonas* spp. can fix nitrogen, conversing N_2 to NH_4^+ under anaerobic
428 conditions [52], which might be the reason for the low NH_4^+ removal during the
429 experiment. Under aerobic conditions, PNSB are unable to grow through the
430 photosynthetic process [53, 54], leading to a general decline in biomass
431 concentrations, (Figure. 2b) and the decrease of relative abundance of
432 *Rhodopseudomonas* spp.. The photobioreactor with PNSB could perform different
433 metabolic pathways depending on light availability and the presence of CO_2 and O_2
434 (Table 3). In anaerobic conditions with light and the existence of both CO_2 and
435 organic carbon source (acetate acid), PNSB prefers to use organic carbon as the
436 carbon source and grows photoheterotrophically. When CO_2 is the sole carbon source,
437 PNSB undergoes the photoautotrophic pathway. While under aerobic conditions,
438 PNSB can consume organic carbon by respiration, and CO_2 will not be consumed.

However, their growth rate under aerobic conditions was significantly lower than under anaerobic conditions [53], which is consistent with this study. This slower growth also stemmed from the competition between PNSB and other heterotrophs, such as *Delftia sp.* and *Microbacterium sp.*, which were better adapted to cope with oxidative stress, giving them a survival advantage under high oxygen conditions. Fig. 8 shows the metabolic pathways of COD removal PNSB under different aeration and light conditions. Aerobic respiration is the main catabolic pathway for organic matter degradation under aerobic conditions, involving three key steps: glycolysis (EMP) or Entner–Doudoroff (ED) pathway, the tricarboxylic acid (TCA) cycle, and the electron transport chain. Under light-anaerobic conditions, energy is generated through photophosphorylation as the main process and substrate-level phosphorylation. The primary catabolic pathways for organic carbon degradation are the EMP/ED pathway, followed by fermentation [55].

4. Conclusions

This work confirmed that PNSB can be quickly enriched under anaerobic conditions and the continuous supply of CO_2. PNSB became predominant within 28 days of operation, even under a light intensity of 200 μmol/m^2/s. *Rhodopseudomonas* spp. was the dominant PNSB species, with a relative abundance of over 90%. The phtobioreactor achieved a removal ratio of COD:N:P of 100:10:2 when no O_2 was supplied. However, when 20.4% O_2 was provided, the removal ratio of COD:N:P was 100:1:1, which highlighted the relevance of the gas mixture fed to the photobioreactor. The continuous supply of O_2 also impacted the abundance of microalgae in the culture, promoting the growth of aerobic heterotrophic bacteria. Removing organic carbon from the synthetic wastewater and keeping CO_2 as a carbon

source in anaeraobic conditions did not change the microbial distribution in the reactor. The removal efficiency of NH_4^+ did not change a lot (26.6% versus 23.7%) with the absence of organic carbon from phase II to phase III. In comparison, the removal efficiency of PO_4^{3-} increased from 11.9% to 27.1%, and the removal efficiency of CO_2 increased from 2.8% to 5.4%. The photobioreactor did not achieve a net CO_2 removal regardless of the experimental conditions tested. A potential strategy to increase CO_2 removal can be an intermittent CO_2 supply in the anaerobic condition during the dark phase without organic carbon. As for the granulation performance, high EPS production was observed, indicating the biomass had the potential to form granules. However, no mature granules formed in the reactor, potentially because of the lack of organic carbon in phase III and the lack of settling time during the whole experiment. Therefore, the operational strategies tested in phase II and phase III in this study could be used to quickly develop PNSB cultures from activated sludge.

For long-term reactor performance, a higher HRT is recommended in the earlier stage to ensure nutrient and COD removal performance. A longer light phase might be helpful for PNSB growth. After the biomass is enriched to a proper concentration, the loading could be increased step by step by reducing HRT. A settling period or other operational conditions could be developed depending on specific research goals, including specific wastewater treatment processes, bioproducts production, and biomass utilization. In this study, each condition was operated for a short period to establish PNSB communities. Further research should assess the impact of various operational factors, such as light conditions, concentrations of CO_2 source, and the influent quality. Additionally, the long-term operation of the system and its stability, as well as the enzymes involved in the metabolism pathways under

different conditions, should be thoroughly investigated.

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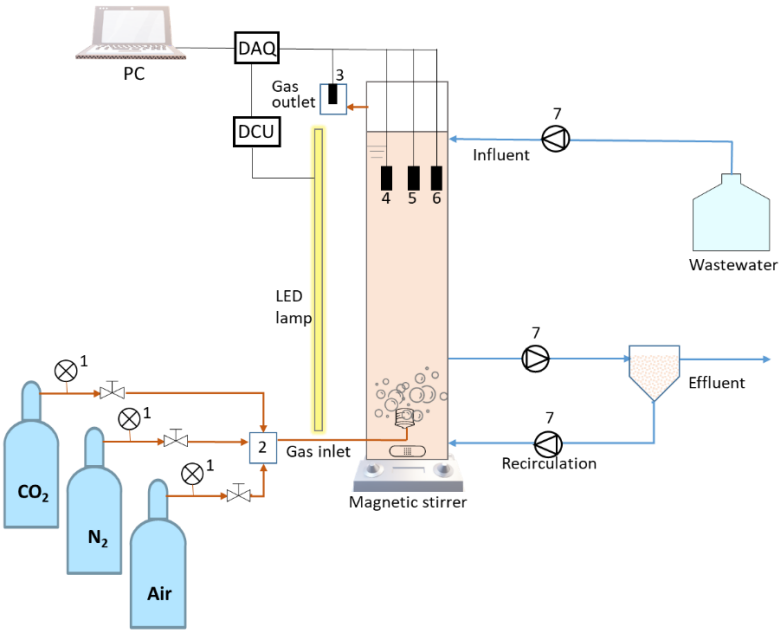


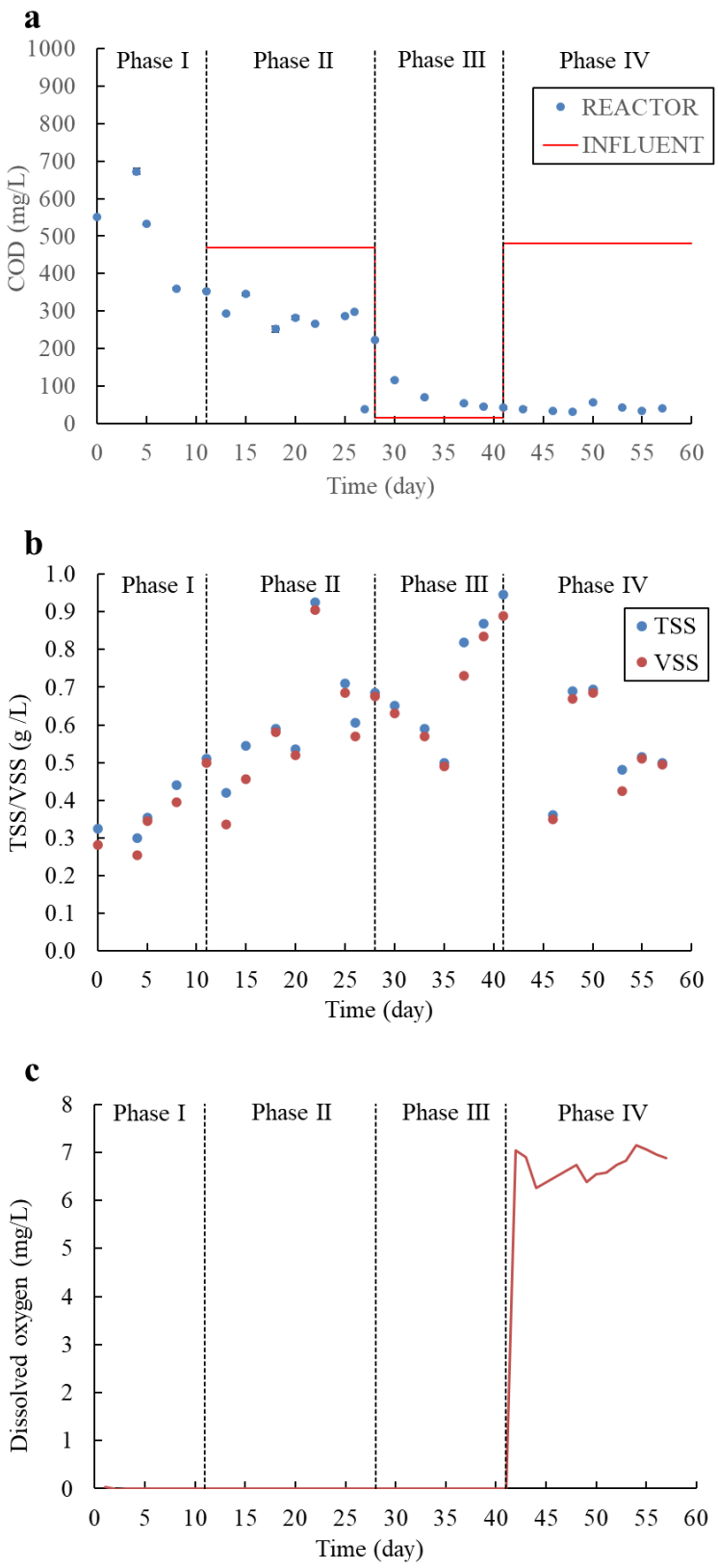
713

714 **Figure 1.** Schematic representation of the experimental setup. DAQ and DCU stand

715 for data acquisition card and digital control unit, respectively; (1) mass flow

716 controller, (2) gas mixing chamber, (3) CO_2 gas sensor, (4) pH sensor, (5) DO sensor,

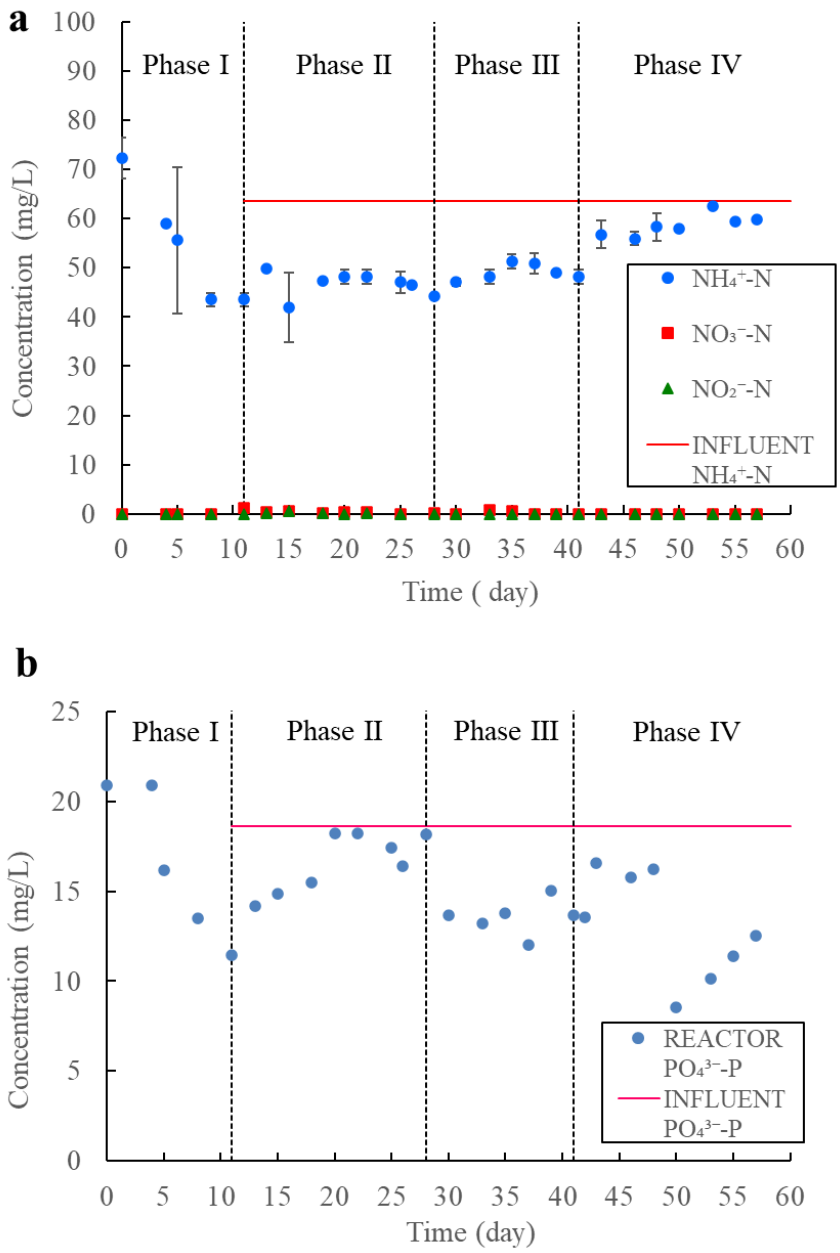
717 (6) temperature sensor, (7) peristaltic pump



718

719 **Figure 2.** Time course of (a) COD, (b) TSS and VSS, and (c) dissolved oxygen

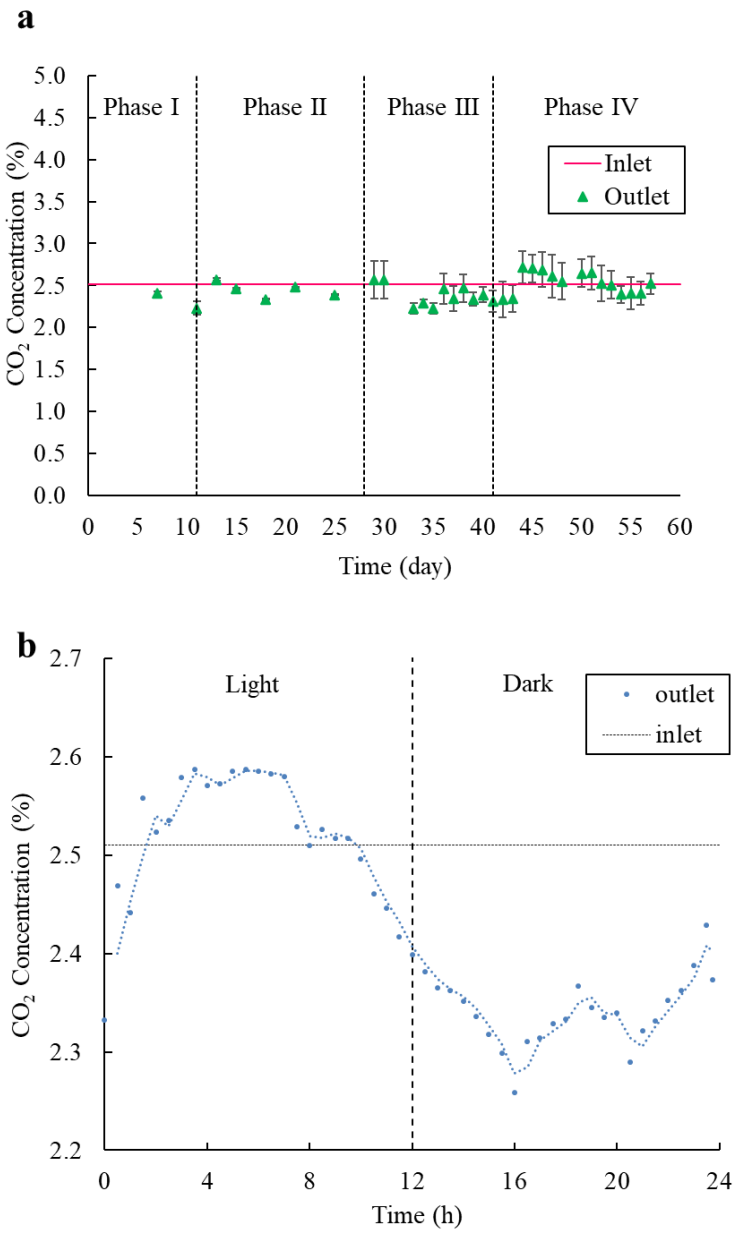
720 concentrations in the four experimental phases tested in the photobioreactor



721

722 **Figure 3.** Time course of (a) inorganic nitrogen species, and (b) PO_4^{3-}-P concentration

723 in the experimental phases tested in the photobioreactor

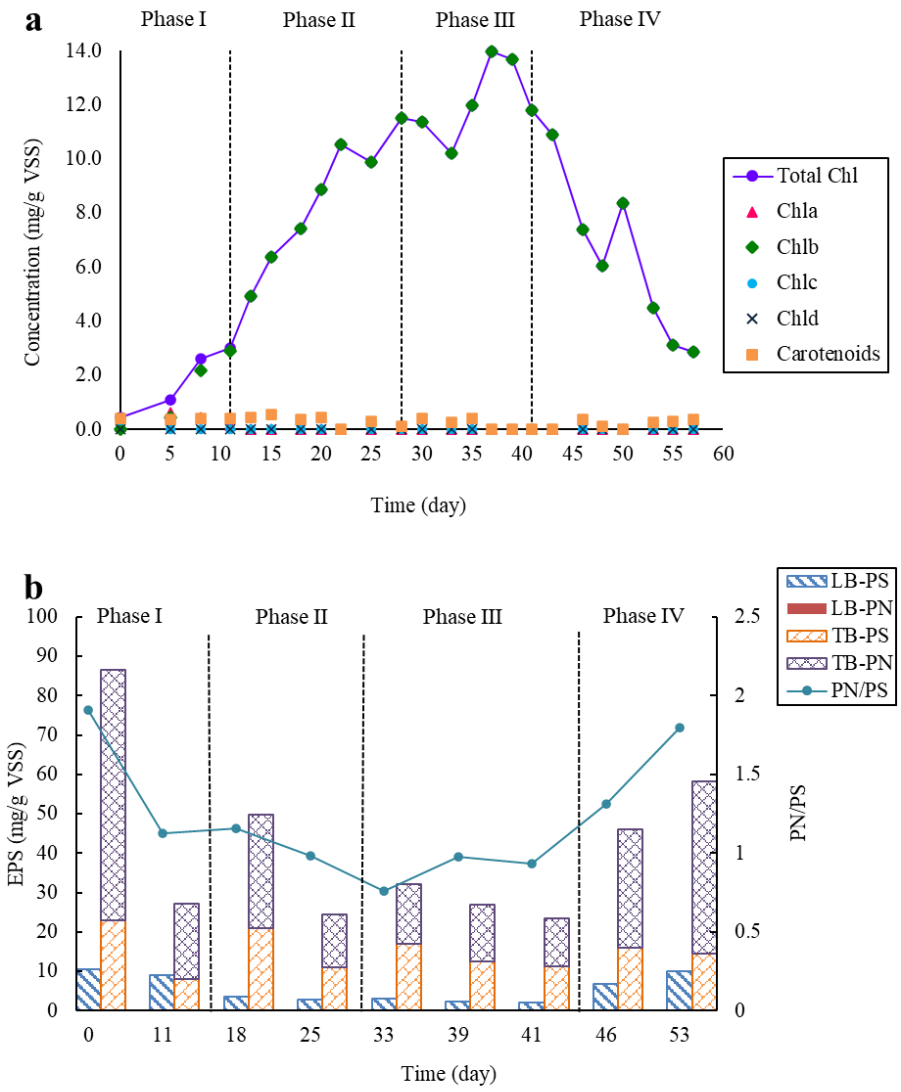


724

725 **Figure 4.** (a) Time course of CO_2 concentration in gas phase in the experimental phases

726 tested in the photobioreactor; (b) One-day dynamics of the CO_2 concentration of

727 experimental phase III (average)



728

729 **Figure 5.** Biomass content of (a) chlorophyll and carotenoids, as well as (b) EPS

730 determined in the experimental phases tested

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732

733

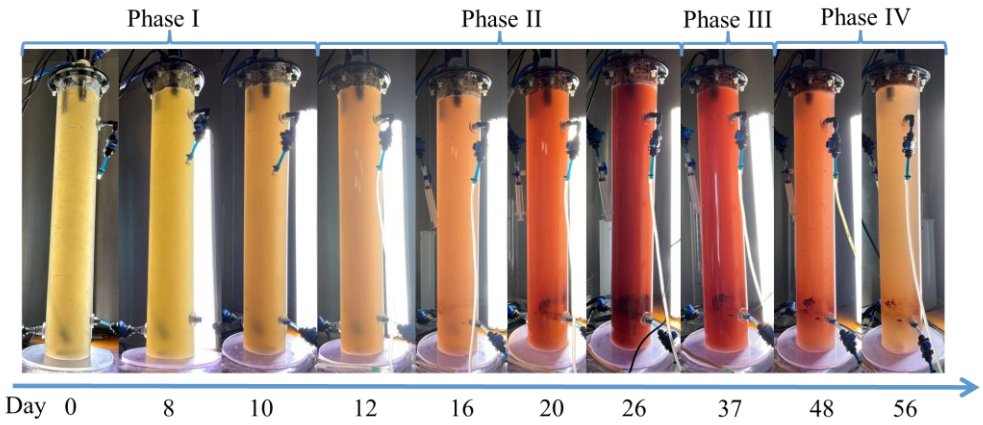
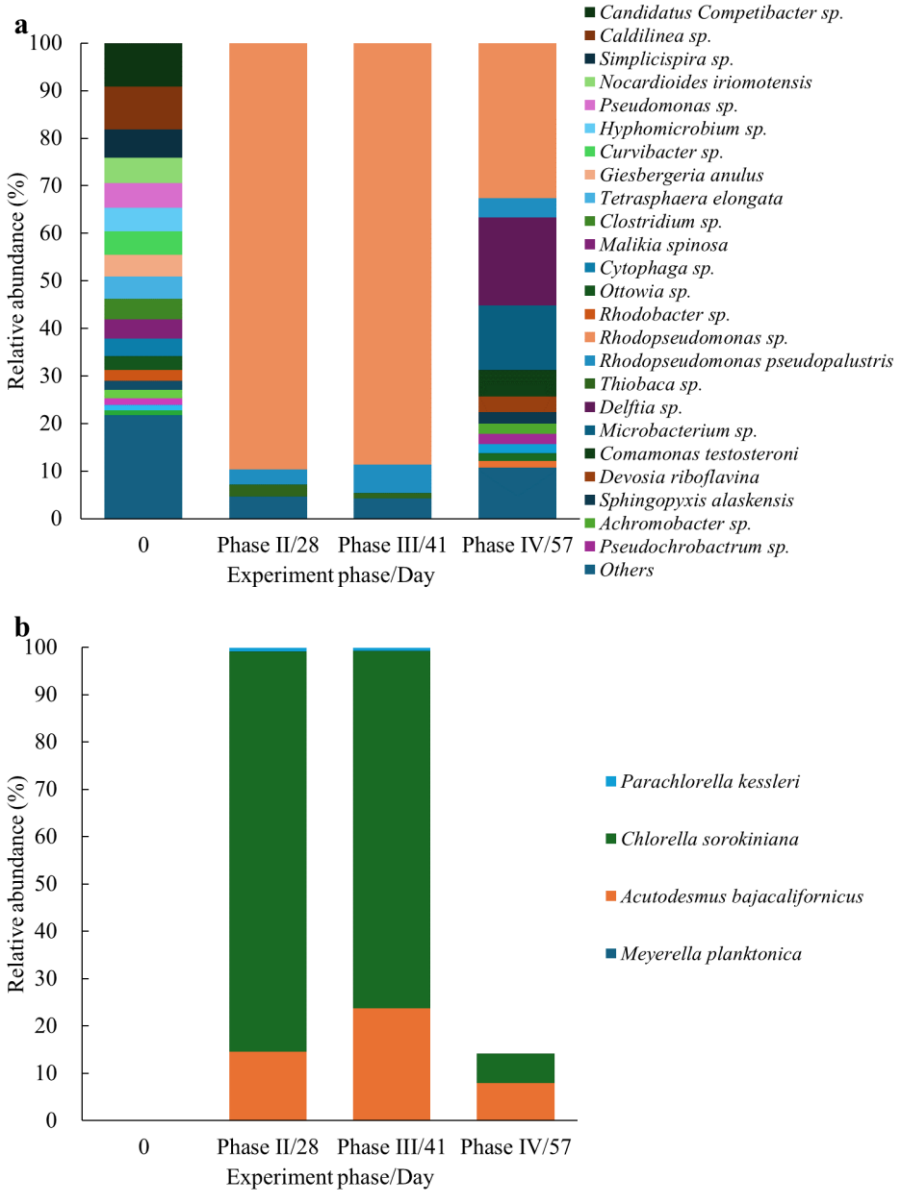


Figure 6. Color changes of the bioreactor in each experimental phase



747

748 **Figure 7.** Microbial community composition at the species level as determined by (a)

749 16S RNA sequencing of biomass; (b) 18S RNA sequencing of biomass (microalgae)

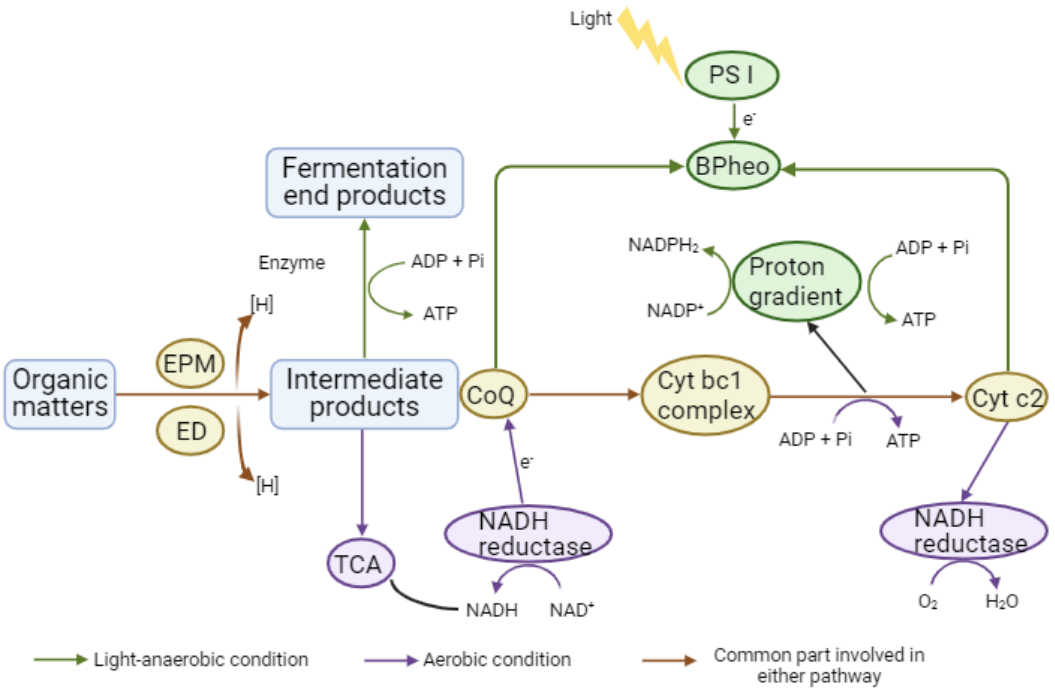


Figure 8. Mechanisms of COD removal in the bioreactor in each condition. Figure was adapted from Lu et al. [55].

761 **Table 1**. Influent COD concentration and composition of the gas fed in each
762 experimental phase.

Experimental phase	**Days**	**HRT (day)**	**Influent COD (mg/L)**	**O_2 in gas phase (%)**	**CO_2 in gas phase (%)**	**N_2 in gas phase (%)**
I	0-11	Batch for liquid phase	469.3±10.3	0	2.5	97.5
II	12-28	2.5	469.3±10.3	0	2.5	97.5
III	29-41	2.5	16±2.8	0	2.5	97.5
IV	42-57	2.5	479.7±10.0	20.4	2.5	76.0

763

Table 2. Average COD, N, and P removal performance achieved in each experimental phase.

Experimental phase	COD			NH_4^+-N			PO_4^{3-}-P			Removal ratio COD:N:P	Biomass specific growth rate (day^{-1})
	Removal efficiency (%)	Removal rate (g COD/(m^3·d))	Specific removal rate (g COD/(kg VSS·d))	Removal efficiency (%)	Removal rate (g N/(m^3·d))	Specific removal rate (g N/(kg VSS·d))	Removal efficiency (%)	Removal rate (g P/(m^3·d))	Specific removal rate (g P/(kg VSS·d))		
I	35.9	18	56.4	39.8	2.62	8.2	20.4	0.9	2.7	100:15:5	0.06
II	36.7±7.5	68.8±15.3	128.7±42.9	26.6±4.4	6.8±1.1	12.9±4.3	11.9±13.2	1.2±1.0	2.5±2.3	100:10:2	0.10
III	NA[a]	NA[a]	NA[a]	23.7±4.1	6.0±1.0	9.4±2.0	27.1±11.4	1.7±0.9	2.7±1.5	-	0.09
IV	91.7±1.6	175.8±3.2	311.8±116.9	10.0±6.7	2.4±1.7	3.9±2.5	29.9±15	2.2±1.2	4.0±2.5	100:1:1	0.07

a. NA: COD was not supplied in this experimental phase.

Table 3. Suggested PNSB metabolic pathway in each experimental phase based on the CO_2 and COD metabolism recorded in the present work.

Experimental phase	**Carbon source**	**Condition of O_2**	**Main metabolic pahtway**	
			Light	**Dark**
II	Organic carbon & CO_2	Anaerobic	Photoheterotrophic	Fermentation/ anaerobic respiration
III	Sole CO_2	Anaerobic	Photoautotrophic	Reductive tricarboxylic acid (rTCA) cycle
IV	Organic carbon & CO_2	Aerobic	Aerobic respiration	Aerobic respiration