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1 **Operational strategies to selectively produce purple bacteria for microbial protein in**
2 **raceway reactors**

3

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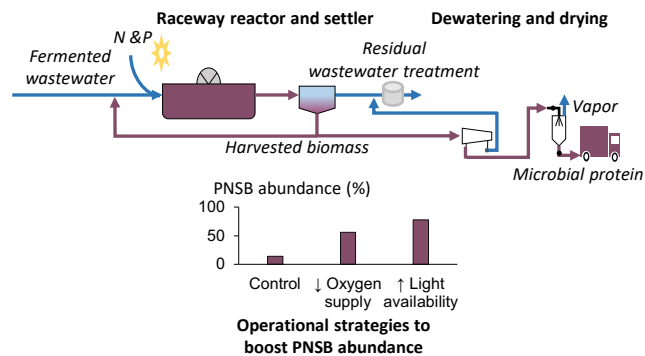
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15 **Graphical abstract**



16

17 **Abstract**

18 Purple non-sulfur bacteria (PNSB) show potential for microbial protein production on
19 wastewater as animal feed. They offer good selectivity (i.e. low microbial diversity and high
20 abundance of one species) when grown anaerobically in the light. However, the cost of closed
21 anaerobic photobioreactors is prohibitive for protein production. While open raceway reactors
22 are cheaper, their feasibility to selectively grow PNSB is thus far unexplored. This study
23 developed operational strategies to boost PNSB abundance in the biomass of a raceway reactor
24 fed with volatile fatty acids. For a flask reactor run at 2-d sludge retention time (SRT), matching
25 the COD-loading rate to the removal rate in the light period prevented substrate availability
26 during the dark period and increased the PNSB abundance from 50-67% to 88-94%. A raceway
27 reactor run at 2-d SRT showed an elevated PNSB abundance from 14% to 56% when oxygen
28 supply was reduced (no stirring at night). The best performance was achieved at the highest
29 surface-to-volume ratio ($10\text{m}^2\text{ m}^{-3}$ increased light availability) showing productivities up to
30 $0.2\text{g protein L}^{-1}\text{ d}^{-1}$ and a PNSB abundance of 78%. This study pioneered in PNSB-based
31 microbial protein production in raceways, yielding high selectivity when avoiding the
32 combined availability of oxygen, COD and darkness.

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36 **Keywords:** Alternative protein source; Single-cell protein, Purple phototrophic bacteria,
37 Anaerobic fermentation; Carboxylate platform; Short-chain fatty acid; High-rate algae pond;
38 Nutrient recovery

39 **1 Introduction**

40 Inefficiencies in the fertilizer-food chain severely distort the carrying capacity of the Earth,
41 surpassing the planetary boundaries (i.e. safe operating space for sustainability) beyond the
42 zone of uncertainty.¹ Mitigation can be brought about by upgrading wastewater resources to
43 microbial protein or single-cell protein, which is the use of microorganisms as an ingredient in
44 animal feed or human food.^{2,3} Protein production from industrial wastewater installations with
45 separated process water and sanitary water is preferred, as European regulation No 767/2009
46 prohibits the use of waste obtained from feces and urine as feed material.⁴ Muys, et al. 2020⁵
47 performed a screening of 19 wastewater installations in the food and beverage industry and
48 observed that 6 companies already separated process water and sanitary wastewater. The study
49 of Muys, et al. 2020⁵ also showed that brewery and dairy wastewater is particularly interesting
50 for protein production as heavy metals in the activated sludge were below legal limits for feed
51 ingredients.

52 Upgrading wastewater resources to microbial protein requires either chemo- or
53 photoheterotrophic microorganisms to convert the organic carbon as well as non-axenic
54 production conditions, as it is cost-wise redundant to sterilize vast amounts of water.² Aerobic
55 heterotrophic bacteria (AHB) grow chemoheterotrophically by making use of oxidation
56 reactions with oxygen as the terminal electron acceptor for energy generation. These bacteria
57 typically have low biomass yields ($0.6 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$) and high growth rates (2-
58 6 d^{-1}).⁶ To date, AHB production on wastewater as a source of microbial protein has already
59 reached full-scale implementation. The company iCell Sustainable Nutrition, for example, has
60 a facility on brewery wastewater in Shanghai (China).^{7,8} However, it is challenging to produce
61 an AHB-based product with low microbial diversity and a high abundance of one dominant
62 species (i.e. microbial selective production). Stability through selectivity in the microbial

63 community may yield stability in nutritional characteristics, yet for AHB this is practically
64 impossible. Aerobic chemoheterotrophy on complex COD mixtures is a widespread metabolic
65 trait. Due to competition, changes in influent and operational conditions shifts in community
66 structure occur.⁹ Photoheterotrophic cultivation of purple non-sulfur bacteria (PNSB) may
67 offer such potential, because of their unique ability to grow highly selectively under anaerobic
68 conditions in the light.¹⁰⁻¹³ PNSB are gram-negative microbes and form a group of purple
69 bacteria, which also comprise the purple sulfur bacteria.¹⁴ PNSB are characterized by high
70 biomass yields (0.9-1.1 g COD_{biomass} g⁻¹ COD_{removed}) and have growth rates between 0.6-3.7 d⁻¹.^{12,15} These yields make their COD usage efficiency higher compared to AHB, yet their
71 substrate uptake rate and, thus the COD removal rate will be lower. In terms of product usage,
72 PNSB have more added-value potential than AHB, which is merely a bulk protein ingredient.
73 Our previous study, for example, has shown that these microbes have antimicrobial properties
74 against shrimp *Vibrio* pathogens.¹⁶ They also contain antioxidant compounds such as
75 carotenoids, which have the potential to stimulate the immune performance of the target
76 animal.¹⁷ However, compared to AHB, there is a lack of full-scale PNSB facilities for microbial
77 protein production. Due to light limitations,¹⁸ lower biomass productivities (0.01-1.13 g
78 COD_{biomass} L⁻¹ d⁻¹)¹⁹ and lower attainable biomass concentrations can be achieved. Hence,
79 larger reactor volumes are required with higher investment costs per volume reactor
80 (photobioreactor i.e. PBR € 1100-5000 m⁻³ vs. aerobic reactor € 300 m⁻³).²⁰⁻²²

82 To achieve selectivity with PNSB (i.e. low microbial diversity and high abundance of
83 one species), current research has focused on closed PBR such as anaerobic membrane
84 bioreactors, anaerobic tubular PBR and illuminated anaerobic sequencing batch reactors.¹⁹
85 These PBR are operated under anaerobic conditions by preventing oxygen entry and only allow
86 the growth of photoheterotrophs and anaerobic chemotrophs. In the case of our previous study
87 on synthetic wastewater, PNSB were able to be selectively produced with an abundance of

88 *Rhodobacter capsulatus* between 93-97% and low microbial diversity (exponent Shannon
89 index between 1.2-1.5).¹³ For microalgae, a review of nine techno-economic analyses on
90 biodiesel production indicated that raceway reactors are more interesting from a cost
91 perspective compared to closed PBR.²³ This is mainly due to the higher investment cost of a
92 vertical tubular PBR, which is between € 1100-5,000 m⁻³ compared to € 14-56 m⁻³ for a
93 raceway reactor.^{20,22} For PNSB, no detailed cost-assessment exists, yet a similar advantage for
94 production in a raceway reactor can be expected.

95 Raceway reactors are easy to operate, maintain and clean.²⁴ These reactors are open
96 systems with a longer light path (e.g. liquid depth of 20 cm vs. 6 cm diameter tubular PBR), a
97 lower lit surface-to-volume ratio of 5 m² m⁻³ (vs. 22 m² m⁻³ tubular PBR) and are agitated with
98 a paddlewheel (vs. circulation pumps in tubular PBR).²⁵ In the context of wastewater treatment,
99 another function is envisaged for PNSB compared to microalgal raceway systems. More
100 specifically, the loading rate, SRT and treatment stage differ. For microalgae, raceway reactors
101 are used for both secondary and tertiary treatment operated at low loading rates (81-388 mg
102 COD L⁻¹ d⁻¹) and long SRT (4-8 d; 31°C).^{26,27} Raceway systems for PNSB, on the other hand,
103 would mainly be used for secondary treatment at higher loading rates and shorter SRT.

104 Raceway reactor could be economically interesting for PNSB yet, achieving selective
105 production is more challenging in these reactors, as air continuously enters the system, which
106 enables the proliferation of competing aerobic heterotrophs (i.e. non-PNSB). Moreover, the
107 oxygen concentration in a raceway reactor is zero due to its direct use as an electron acceptor,
108 making the growth of anaerobic chemotrophs such as acidogenic microorganisms and sulfate-
109 reducing bacteria (SRB) also possible. According to the authors' knowledge, research on PNSB
110 production in raceway reactors is limited. One conference abstract by Fradinho, et al. 2019²⁸
111 focuses on polyhydroxyalkanoate production with PNSB in raceway reactors, yet specific
112 operational conditions are not fully described. It can, however, be hypothesized that the

113 following operational strategies are essential to maximize PNSB selectivity (i.e. increase PNSB
114 abundance and decrease Shannon diversity index): (i) limiting the oxygen supply may decrease
115 the growth of aerobic chemotrophs, (ii) increasing the light availability or the illumination
116 period may aide PNSB in their competition for COD with aerobic chemotrophs and anaerobic
117 chemotrophs such as acidogenic microorganisms and SRB, (iii) short sludge retention times
118 (SRT) may washout slower-growing microorganisms such as microalgae, and (iv) matching
119 the COD-loading rate to the achievable removal rate in the light period prevents substrate
120 availability during the dark period and may decrease the competition with (an)aerobic
121 chemotrophs.

122 This study hypothesized that PNSB can be selectively produced in a raceway reactor
123 provided a good combination of operational strategies. The first objective was to assess the
124 metabolic growth modes of PNSB and non-PNSB. This would enable us to elucidate how the
125 individually metabolic growth modes of PNSB and non-PNSB contribute in a raceway reactor.
126 The second objective was to study the effect of reactor-specific operational strategies such as
127 oxygen supply, light availability, SRT and COD-loading on the PNSB abundance and
128 microbial diversity.

129 **2 Materials and methods**

130 **2.1 Inocula and medium**

131 An *Rb. capsulatus* strain, isolated in our previous study,¹² was used as model PNSB for the
132 axenic flask and non-axenic raceway reactor experiments. This species was selected based on
133 a prior evaluation made between five PNSB cultures, where it showed to have the highest
134 photoheterotrophic growth rate on synthetic wastewater. This species is able to grow photo-
135 and chemoheterotrophically,¹⁰ which enables examination of different PNSB growth kinetics

136 in raceway reactors. Aerobic activated sludge of a local brewery company (AB InBev,
137 Belgium, Leuven) was used as a proxy for a non-PNSB inoculum.

138 A synthetic medium was chosen over real wastewater to avoid inherent variability from
139 real wastewater and to clearly discriminate the effect of specific operational strategies on the
140 microbial community. Volatile fatty acids (VFA) were used as carbon source as a proxy for
141 fermented wastewater, as we argued in a previous study that fermentation prior to protein
142 production will favor the microbial selectivity.^{2,12} The COD concentration was 3 g L⁻¹ and
143 contained a defined mixture of acetate, propionate and butyrate on a 1/1/1 COD basis. The
144 medium also contained 0.8 g L⁻¹ KH₂PO₄, 0.5 g L⁻¹ MgCl₂·6H₂O, 0.1 g L⁻¹ CaCl₂·2H₂O, 0.7 g
145 L⁻¹ Na₂SO₄, 1.2 g L⁻¹ NH₄Cl, 1.0 g L⁻¹ NaCl and 0.3 g L⁻¹ NaHCO₃. 1 mL of trace elements
146 and 1 mL of vitamin solution, based on the composition of Imhoff 2006¹⁰, was also added per
147 liter of water. PNSB grown photoheterotrophically on this VFA mixture have a biomass yield
148 that approximate 1 g COD_{biomass} g⁻¹ COD_{removed}.^{12,29} This makes it easy to assess the
149 chemoheterotrophic growth of PNSB and of competing non-PNSB, as a lower biomass yield
150 implies oxidation of COD to CO₂. Note that the overall biomass yield in a treatment system
151 will be lower than 1 as COD removed during the anaerobic fermentation will also be included.

152 **2.2 Overview of the experiments**

153 Three sets of flask and raceway reactor experiments were performed in this study to explore
154 the metabolic growth modes of PNSB and the effect of oxygen supply, light availability and
155 SRT on PNSB selectivity (Table 1).

156

157 **Table 1** Objectives and experimental setup of three tests to grow a protein-rich PNSB biomass on brewery wastewater. *Rhodobacter capsulatus* was used as purple non-sulfur bacterium (PNSB)
 158 inoculum and aerobic brewery sludge as non-PNSB inoculum. Experiments were performed at 28°C. Surface-to-volume (S/V) ratios were calculated based on the illuminated surface area. The
 159 flasks were illuminated from the side and the raceway reactor from the top. The axenic experiments were performed with a sterile medium inoculated with *Rb. capsulatus* and samples were taken
 160 axenically. The non-axenic experiments were conducted with a non-sterile medium and inoculated with a community enriched in *Rb. capsulatus*. Dissolved oxygen (DO) concentrations are shown
 161 as averages with standard deviations. The determined oxygen mass transfer coefficients ($k_L a$) for the flasks filled with a working volume of 500 mL, flasks with a working volume of 200 mL, and
 162 raceway reactor were 0.3 h⁻¹, 1.8 h⁻¹, and 1.2 h⁻¹, respectively. The oxygen transfer rates (OTR) were calculated as $k_L a \times (DO_S - DO)$. DO_S: DO saturation level (7.85 mg L⁻¹ at 28°C); HRT: hydraulic
 163 retention time

Objective	DO (mg O ₂ L ⁻¹)	OTR (mg O ₂ L ⁻¹ d ⁻¹)	Stirring (on/off)	Illumination (light/dark)	S/V ratio (m ² m ⁻³)	HRT (d)	Inoculum	Cultivation	Reactor type	
Assess metabolic growth modes of PNSB and non-PNSB (section 3.1)	N.D.*	331**	24h/0h	24h	61***	Batch****	PNSB	Axenic	Flask	
	-	0		24h						
	N.D.*	331**		0h						
	-	0		0h						
	N.D.*	331**		0h						
	-	0		0h						
Effect of SRT on PNSB growth (section 3.2)	0.18 ± 0.04	331	24h/0h	12h/12h	61	1.25	PNSB	Non-axenic	Flask	
	0.18 ± 0.06	331								2
	0.15 ± 0.04	333								3
PNSB selectivity over sequential batches (section 3.3)	0.17 ± 0.15	219	24h/0h	12h/12h	5****	2	PNSB	Non-axenic	Raceway	
	0.14 ± 0.12	220	12h/12h							5
	0.24 ± 0.12	217	24h/0h							10

164 *DO was not determined (N.D.) during the axenic experiments as it would result in contamination.

165 **The DO concentration for the OTR calculation was assumed to be 0.18 mg O₂ L⁻¹.

166 ***The surface area exposed to air for the flask experiments was equal to 11 m² m⁻³ and for the raceway reactor 5-10 m² m⁻³.

167 ****Growth experiment between 50-150h, stopped when the stationary phase was reached

168 **2.2.1 Assess the metabolic growth modes of PNSB and non-PNSB**

169 Flask batch experiments were performed to explore the photoheterotrophic and (an)aerobic
170 chemoheterotrophic metabolic growth mode of PNSB along with the (an)aerobic
171 chemoheterotrophic metabolic growth mode of competing non-PNSB. These tests were
172 conducted to understand how these growth modes may individually contribute in a raceway
173 reactor.

174 To explore the metabolic growth modes of PNSB, four different conditions were tested
175 under axenic conditions: (i) illumination with oxygen supply to study the combined photo- and
176 chemotrophic growth (conditions prevalent in a raceway reactor), (ii) illumination without
177 oxygen supply to study the phototrophic growth, (iii) no illumination with oxygen supply to
178 study the aerobic chemotrophic growth (i.e. aerobic growth) not to be confused with aerobic
179 growth of PNSB on hydrogen gas as an electron donor (i.e. aerobic chemoautotrophic growth)
180 and (iv) no illumination without oxygen supply to study the anaerobic chemotrophic growth
181 (i.e. acidogenic metabolism). *Rb capsulatus* was first axenically pre-cultivated in a climate
182 chamber (Snijders Scientific) with the pre-autoclaved VFA medium (section 2.1). Flasks of
183 500 mL were then filled with 200 mL of autoclaved VFA medium and inoculated with *Rb.*
184 *capsulatus* at an initial total suspended solids (TSS) concentration of 0.02 g L⁻¹. All
185 experiments were tested in triplicate. A detailed description of the methodology can be found
186 in Supporting Information S1.

187 An experiment was also performed to assess the effect of oxygen supply on the photo-
188 and chemoheterotrophic growth of PNSB. The methodology and results are available in
189 Supporting Information S2-S3.

190 **2.2.2 Effect of SRT and COD-loading rate on PNSB growth**

191 These experiments were performed to explore the effect of SRT on productivity, biomass yield,
192 biomass composition and PNSB selectivity.

193 Experiments were performed under combined photo- and chemotrophic conditions,
194 allowing the entry of oxygen along with illumination (i.e. conditions prevalent in raceway
195 reactor). Flasks of 500 mL were used as reactors and illuminated through a natural 12-h
196 light/12-h dark regime with two halogen lamps at a light intensity of 30 W m^{-2} (vs. previous
197 flask experiments section 2.2.1: 24-h light or 24-h dark). The flasks were filled with 200 mL
198 of medium (COD concentration 3 g L^{-1} see section 2.1) corresponding to a maximum OTR of
199 $336 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$. An influent COD concentration of 6 g L^{-1} was also tested to study the effect
200 of a higher COD-loading rate on the microbial selectivity (more COD available during the
201 night). The experiment was performed non-axenically with *Rb. capsulatus* as initial inoculum.
202 The tested SRT were 1.25 d, 2 d and 3 d. The hydraulic retention time was equal to the SRT.
203 All tests were performed in biological duplicate. A detailed description of the methodology can
204 be found in Supporting Information S4.

205 **2.2.3 Operational strategies to steer PNSB selectivity and reactor performance**

206 A final experiment was performed to demonstrate that PNSB can be maintained in a raceway
207 reactor over multiple generations and determine the best operational strategy in terms of
208 productivity and COD removal. The productivity was calculated by dividing the biomass
209 concentration or protein concentration by the SRT.

210 A 100-L raceway reactor was used under non-axenic conditions (MicroBio Engineering
211 Inc., USA) with *Rb. capsulatus* as inoculum. The reactor was filled with the VFA-based
212 medium (section 2.1). Three operational strategies were tested: (i) 12-h light/12-h dark with
213 24-h stirring at a surface-to-volume ratio of $5 \text{ m}^2 \text{ m}^{-3}$ as a benchmark (reactor filled up to 100
214 L and depth 20 cm) with a duration of 9.6 d or 4.8 times the SRT, (ii) 12-h light/12-h dark with
215 12-h stirring during the light period at a surface-to-volume ratio of $5 \text{ m}^2 \text{ m}^{-3}$ with a duration of
216 9.5 d or 4.7 d the SRT to study the effect oxygen supply and (iii) 12-h light/12-h dark with 24-
217 h stirring at a surface-to-volume ratio of $10 \text{ m}^2 \text{ m}^{-3}$ (higher light availability vs. $5 \text{ m}^2 \text{ m}^{-3}$ reactor

218 filled up to 50 L and depth 10 cm) with a duration of 15 d or 7.5 times the SRT to study the
219 effect of light. The SRT was chosen based on the maximum specific growth rate during the
220 batch experiments (Supporting Information S5-S6). The microbial community structure can be
221 different in a single batch compared to an experiment over multiple SRT, which can also affect
222 the growth kinetics. For safety reasons, an SRT of 2 d was chosen for the three conditions to
223 prevent washout from the reactor. The effluent was first removed and influent was then added
224 before the start of the light period (i.e. sequencing batches). A detailed description of the
225 methodology can be found in Supporting Information S7.

226 **2.3 Analytical procedures**

227 During the experiments, TSS, volatile suspended solids, COD, sulfate, ammonium, nitrite,
228 nitrate, ortho-phosphate, protein and bacteriochlorophyll a were analyzed. A detailed
229 description of the analytic procedures can be found in Supporting Information S8. The oxygen
230 mass transfer coefficient (k_{La}) was derived from the increasing DO levels after chemical
231 deoxygenation with sodium sulfite.³⁰ The k_{La} for the different flask and raceway reactors are
232 presented in Table 1 along with the DO concentration measured during the experiments. The
233 OTR was calculated by multiplying k_{La} with the difference between the DO saturation
234 concentration (i.e. 7.85 mg L⁻¹ at 28°C) and the actual DO concentration.

235 **2.4 Microbial community analyses**

236 Genomic DNA was extracted from biomass samples collected (after steady-state) across the
237 reactor experiments using the DNeasy UltraClean microbial extraction kit (Qiagen, Venlo, the
238 Netherlands) according to the manufacturer's instructions. In brief, the V3-V4 hypervariable
239 region of the bacterial 16S rRNA gene pool of the DNA extracts was amplified by PCR using
240 the pair of 341f/806r primers prior to sequencing of PCR products using a HiSeq 2500
241 sequencer (Illumina, USA). The data have been deposited with links to BioProject accession

242 number PRJNA720505 in the NCBI BioProject database. A detailed description of the wet-lab
243 and dry-lab workflows can be found in Supporting Information S9.

244 **2.5 Statistical analyses**

245 Statistical analyses were performed in R (version 3.4.1) using RStudio (RStudio®, USA) for
246 Windows.³¹ Student's t-test were conducted to compare means. Normality of data residuals
247 was tested using the Shapiro-Wilk normality test. The assumption of homoscedasticity was
248 verified through a Levene's test. The non-parametric Kruskal-Wallis rank sum test was
249 executed when normality was rejected. The Welch's t-test was used in case of
250 heteroscedasticity. A significance level of $p < 0.05$ was chosen.

251 The maximum specific growth rate in section 3.1 was calculated through the modified
252 Gompertz equation by using the GraphPad Prism version 5.03 for Windows.³²

253 **3 Results and discussion**

254 **3.1 Assess the metabolic growth modes of PNSB and non-PNSB**

255 Axenic batch experiments in flask bottles were performed to determine how the different
256 metabolic growth modes of PNSB and non-PNSB separately would contribute to the joint
257 process in a raceway reactor. Four conditions were tested. The first test used passive aeration
258 and illumination. This mimics the conditions prevalent in a raceway reactor and, thus, allows
259 for combined photo-and chemoheterotrophic growth. The other three batch tests explored the
260 individual metabolic growth modes.

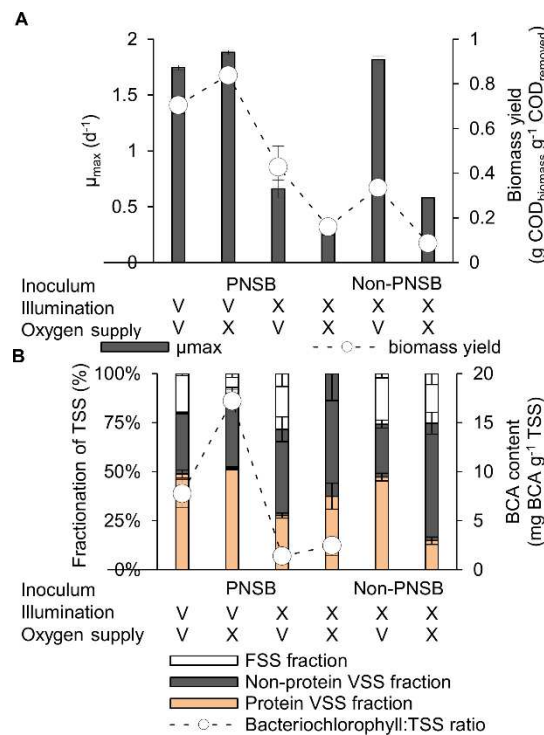
261 The results of the batch growth experiments (Figure 1) show that during the combined
262 photo- and chemotrophic growth (i.e. light and oxygen supply), the phototrophic metabolism
263 (i.e. under light) was dominant and not their chemotrophic metabolism (i.e. oxygen supply).
264 Biomass yields for their phototrophic and the combined photo- and chemotrophic metabolisms
265 were similar ($p > 0.05$) and growth rates were almost equal. There was, therefore, more photo-

266 assimilation of COD than oxidation to CO₂. The bacteriochlorophyll content was lower for the
267 combined photo- and chemotrophic metabolism than for the phototrophic metabolism, yet still
268 6 times higher than for the aerobic chemotrophic growth (i.e. no illumination with oxygen
269 supply). It should be noted that the chemotrophic metabolism of PNSB contributes more to
270 growth when the oxygen supply increases. Supporting Information S3 shows that growth rates
271 for the combined photo- and chemotrophic metabolism do not change, yet the aerobic
272 chemotrophic growth increases. This implies that chemotrophic growth contributed more to
273 the combined photo- and chemotrophic metabolism at a higher oxygen supply. It remains,
274 however, unclear whether the same PNSB species performs both metabolisms simultaneously
275 or if there is a division of labor between bacteria. More research is needed to elucidate this.

276 In a full-scale raceway reactor, the DO concentration will approximate zero due to the
277 direct consumption of oxygen, allowing anaerobic fermentation of COD. The anaerobic
278 chemotrophic growth of PNSB was, therefore, tested with a more complex medium of 3.7 g
279 COD L⁻¹ containing a mixture of organics such as yeast, peptone, malt extract, acetate,
280 propionate and butyrate (details Supporting Information S1). PNSB were able to anaerobically
281 ferment organics (Figure 1). Growth rates and biomass yields were respectively $0.3 \pm 0.08 \text{ d}^{-1}$
282 and $0.16 \pm 0.09 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$, lower than their combined photo- and
283 chemotrophic growth ($1.75 \pm 0.05 \text{ d}^{-1}$; $0.71 \pm 0.07 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$). A similar
284 observation was made by Schultz Weaver 1982³³ with low anaerobic growth rates and biomass
285 yields for *Rb. capsulatus* ($\approx 0.08 \text{ d}^{-1}$; $0.09 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$) and *Rhodospirillum*
286 *rubrum* ($\approx 0.13 \text{ d}^{-1}$; $0.11 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$). The non-PNSB inoculum showed growth
287 rates of $0.58 \pm 0.03 \text{ d}^{-1}$ or 2 times higher compared to PNSB. Therefore, anaerobic fermentation
288 will mainly be performed by competing non-PNSB, which can act as symbionts by making
289 COD available for PNSB. Stronger competition during the light and dark period might arise
290 from aerobic chemotrophic non-PNSB since their growth rate was 2.8 times higher than for the

291 aerobic chemotrophic growth of PNSB and equal to the combined photo- and chemotrophic
 292 growth ($p > 0.05$).

293 PNSB cells were able to produce bacteriochlorophyll a during the aerobic and anaerobic
 294 batch test in the dark and in the light (Figure 1A) because the activation of photosynthetic genes
 295 is dependent on the oxidative conditions.³⁴ Ghosh, et al. 1994³⁵, for example, found that the
 296 formation of photosynthetic membranes is triggered at DO concentrations lower than 0.40 mg
 297 $O_2 L^{-1}$.



298 **Figure 1** (A) maximum specific growth rate and biomass yield for purple non-sulfur bacteria (PNSB) and non-PNSB along
 299 with (B) biomass fractionation and bacteriochlorophyll a (BCA) content. Tested conditions: combined photo- and
 300 chemoheterotrophic (illumination: V; oxygen supply: V), photoheterotrophic (V; X), aerobic chemoheterotrophic (X; V) and
 301 anaerobic chemoheterotrophic (X; X) growth. The oxygen transfer rate was 336 mg $O_2 L^{-1} d^{-1}$. Experiments were performed
 302 axenically with *Rhodobacter capsulatus* used as model PNSB. Non-PNSB were grown non-axenically. Averages with standard
 303 error. TSS: total suspended solids; VSS: volatile suspended solids; FSS: fixed suspended solids i.e. ash
 304

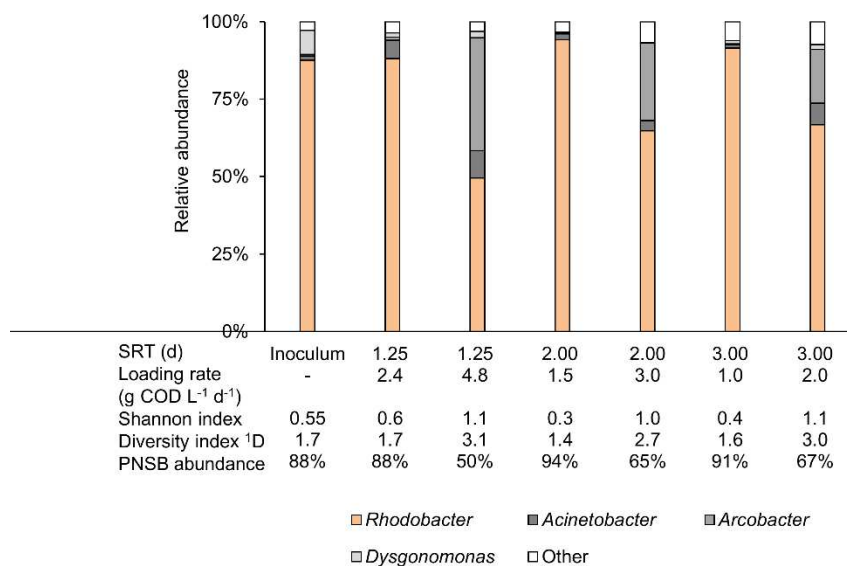
305 3.2 Effect of SRT and COD-loading rate on PNSB growth

306 Sludge age is an important parameter in open community biotechnology as it can impose
 307 selective pressure on the microbial community. Slow-growing microbes are washed out, while

308 faster growers are retained. This continuous experiment was performed under non-axenic
 309 conditions in flask bottles to study the effect of SRT on PNSB selectivity.

310 Overall, PNSB abundances did not show substantial differences between SRT (Figure
 311 2). This is in line with our previous observation where we tested the effect of SRT on PNSB
 312 abundance in a closed PBR.¹² If the reactor is overloaded, PNSB will not be able to remove all
 313 COD during the light period, which can lead to increased growth of competing chemotrophs.
 314 A lower microbial selectivity was observed for all tested SRT when the COD-loading rate was
 315 doubled (Figure 2). The relative PNSB abundance decreased from 88-94% to 50-67% and the
 316 Shannon diversity index increased from 0.4-0.6 to 1.0-1.1, indicating a lower microbial
 317 selectivity. The abundance of the (an)aerobic chemoheterotrophs *Arcobacter* was
 318 predominantly higher at higher loading rates (17-36%). Hence, COD availability during the
 319 night will negatively influence the PNSB abundance in reactors operated in batch mode. We
 320 envision that a full-scale raceway reactor will be operated in sequential batch mode. This
 321 implies that fresh wastewater is only added during the light period and not during the dark
 322 phase to prevent substrate availability for competing microbes.

323 Comparing light with dark periods, the COD removal rates did not change (Supporting
 324 Information S10). The highest rates were observed at the lowest SRT (1.25 d).



325

326 **Figure 2** Effect of sludge retention time (SRT) on microbial community composition, Shannon's H index, $\exp(H')$ and purple
327 non-sulfur bacteria (PNSB) abundance. Flasks were used as a reactor. The PNSB genera *Rhodobacter* and *Rhodopseudomonas*
328 are all marked in orange. Samples obtained after 2-10 SRT.

329 **3.3 Reactor performance and community dynamics over sequential batches**

330 This continuous experiment was conducted under non-axenic conditions to demonstrate that
331 PNSB can be maintained in a raceway reactor over multiple generations (4.7-7.5 times the
332 SRT), investigate the effect of light, oxygen supply and the combination of both on PNSB
333 selectivity and determine the best operational strategy in terms of productivity and COD
334 removal.

335 The highest productivities ($0.21 \text{ g protein L}^{-1} \text{ d}^{-1}$ corresponding with $0.43 \text{ g TSS L}^{-1} \text{ d}^{-1}$;
336 Figure 3) and removal rates ($0.79 \text{ g COD L}^{-1} \text{ d}^{-1}$) were achieved when PNSB were most
337 abundant (Figure 4). This was during the experimental condition of 24-h stirring and 12-h
338 light/12-h dark at the highest surface-to-volume ratio of $10 \text{ m}^2 \text{ m}^{-3}$. A higher ratio of $10 \text{ m}^2 \text{ m}^{-3}$
339 increased the light availability, resulting in higher biomass concentrations ($0.81 \pm 0.04 \text{ g TSS}$
340 L^{-1}) relative to the benchmark of $5 \text{ m}^2 \text{ m}^{-3}$ ($0.62 \pm 0.02 \text{ g TSS L}^{-1}$). For a closed PBR operated
341 on the same medium at an SRT of 1 d (vs. 2 d for raceway reactor), we reached TSS
342 productivities that were 1.5-2.6 times higher compared to the raceway reactor.¹² This was
343 probably due to the higher light availability in the PBR compared to a raceway reactor (surface-
344 to-volume ratio 33 vs. 5-10 $\text{m}^2 \text{ m}^{-3}$). Capson-Tojo, et al. 2020¹⁹ have compared the biomass
345 productivity of several PNSB reactors. The productivity for closed PBR were maximally 0.77
346 $\text{g TSS L}^{-1} \text{ d}^{-1}$ (10-39°C), 1.8 times higher than the productivity in this study (28°C). For the
347 COD removal rate, values between $0.45\text{-}0.79 \text{ g COD}_{\text{removed}} \text{ L}^{-1} \text{ d}^{-1}$ were reached in the raceway
348 reactor, comparable with the median productivity of photo anaerobic membrane bioreactors
349 ($0.96 \text{ g COD}_{\text{removed}} \text{ L}^{-1} \text{ d}^{-1}$, 10-39°C). For the microalga *Chlorella vulgaris* cultivated in the
350 same reactor (12-h light/12-h dark), the productivity was $0.009 \text{ g protein L}^{-1} \text{ d}^{-1}$ or 22 times
351 lower compared to PNSB.³⁶ This was probably due to the higher growth rates of PNSB of 0.6-

352 3.7 d⁻¹ compared to the ones of microalgae of 0.60-1.38 d⁻¹ (28°C).^{12,37,38} This is also true for
353 microalgal bacterial floc technology, a wastewater treatment system where microalgae supply
354 oxygen to aerobic bacteria for COD removal.²⁷ Van Den Hende, et al. 2014²⁶ observed biomass
355 productivities and COD removal rates of respectively 0.07-0.22 g TSS L⁻¹ d⁻¹ and 0.06-0.30 g
356 COD_{removed} L⁻¹ d⁻¹ with the microalgal bacterial floc technology (31°C), lower than the PNSB
357 raceway reactor operated in this study (0.24-0.43 g TSS L⁻¹ d⁻¹ and 0.45-0.79 g COD_{removed} L⁻¹
358 d⁻¹; 28°C). The aerobic bacteria in the microbial community could theoretically cope with low
359 SRT, yet the system is operated at an SRT of 4-8 d to maintain microalgae in the reactor (vs. 2
360 d SRT in this study).

361 Biomass yields increased around 1.2 times when stirring was prevented during the night
362 or at higher surface-to-volume ratios (Figure 3). The yields in the reactor were lower than the
363 theoretical yield for anaerobic photoheterotrophic growth (1 g COD_{biomass} g⁻¹ COD_{removed}) as
364 PNSB and non-PNSB aerobically oxidize COD. The same was observed for the axenic flask
365 experiment (Figure 1), where the biomass yield was 0.71 g COD_{biomass} g⁻¹ COD_{removed} for the
366 combined photo- and chemotrophic condition.

367 The bacteriochlorophyll a content of the biomass was between 6-10 mg g⁻¹ TSS (Figure
368 3). *Rb. capsulatus* grown anaerobically in the light typically has values around 21-50 mg g⁻¹
369 TSS.³⁹ The lower pigment content in the raceway reactor compared to strict anaerobic systems
370 was due to aerobic chemoheterotrophic growth of PNSB. Ghosh, et al. 1994³⁵ have shown that
371 PNSB grown heterotrophically with oxygen can produce pigments at DO concentrations lower
372 than 0.40 mg O₂ L⁻¹. We observed that the bacteriochlorophyll a content of *Rb. capsulatus* was
373 1.4 mg g⁻¹ TSS or 12 times lower compared to their anaerobic photoheterotrophic growth
374 (Figure 1A). Low pigmentation was also visually observed for aerobically grown *Rb.*
375 *sphaeroides*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* in our previous
376 study.¹³ It was, thus, likely that the overall bacteriochlorophyll a content of the biomass

377 produced in a raceway reactor was lower compared to strict anaerobic conditions due to the
378 contribution of aerobic heterotrophic growth to biomass production.

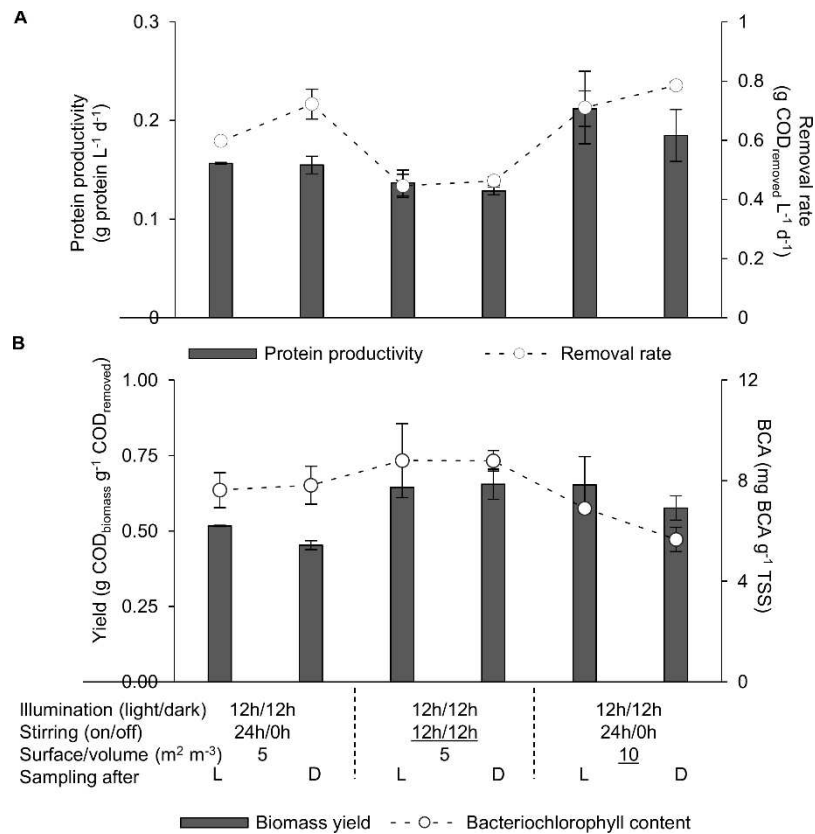
379 In terms of PNSB selectivity (Figure 4), preventing the combination of oxygen supply
380 and darkness (not stirring) was an effective strategy. The PNSB abundance was 56% (vs. 14%
381 benchmark 24-h stirring) and the microbial community had a higher diversity showing a lower
382 exponent of the Shannon diversity index (3.7 vs. 4.3 for benchmark). The decrease in PNSB
383 abundance to 41% was notable during the dark period along with the increase of the exponent
384 of the Shannon diversity index from 3.7 to 7.2. This drop was due to a lower contribution of
385 PNSB to biomass production relative to non-PNSB. In the dark period, PNSB only use their
386 chemotrophic growth mode, which has a lower maximum specific growth rate compared to the
387 combined photo- and chemotrophic growth mode (see Figure 1). The reactor operated with 24-
388 h stirring at a surface-to-volume ratio of $10 \text{ m}^2 \text{ m}^{-3}$ was the best strategy in terms of PNSB
389 selectivity, showing a PNSB abundance of 78% or 5.6 times higher compared to the benchmark
390 and very comparable to the inoculum. The exponent of the Shannon diversity index was only
391 2.5, the lowest for all conditions and even lower than the inoculum (3.5). This increase in PNSB
392 abundance was also notable from the bacteriochlorophyll absorbance ratio of 800:660 nm and
393 860:660 nm, which were 1.2-1.3 times higher compared to the benchmark (Supporting
394 Information S11). It was likely that the effect of higher light availability was stronger than the
395 negative effect of an increased oxygen transfer rate. This implies that light availability is key
396 to boost PNSB selectivity in a raceway reactor. The findings also show that a raceway reactor
397 can approach the PNSB selectivity of a closed PBR. Potential higher PNSB abundances might
398 even be achieved if oxygen supply is prevented during the night along with high surface-to-
399 volume ratios. This can be achieved by stopping the paddlewheel during the night and
400 decreasing the water depth of the reactor. Raceway reactors typically have a water depth of

401 around 20 cm.²⁵ A cost-benefit analysis is, therefore, still required to understand whether an
402 increased productivity could justify a higher investment cost.

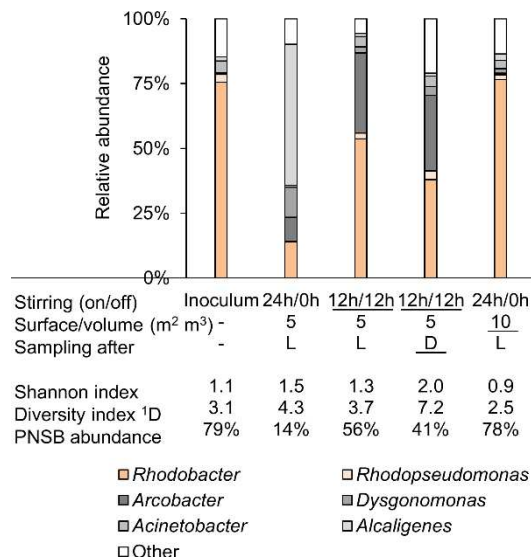
403 Other PNSB genera were also present in the system such as *Rhodopseudomonas* (2-3%),
404 and *Blastochloris* (< 0.2%). The main competing genera were *Acinetobacter* (aerobic
405 chemoheterotroph), *Dysgonomonas* (anaerobic chemoheterotrophs), *Arcobacter* ((an)aerobic
406 chemoheterotrophs) and *Alcaligenes* (aerobic chemoheterotrophs) with an abundance of
407 respectively 1-4%, 1-12%, 0-31% and 1-54%. Microalgae are undesirable due to oxygen
408 production and stimulation of aerobic chemotrophic COD conversion. These microbes were
409 not detected through the absorbance spectra (no chlorophyll peaks) and no cyanobacteria were
410 identified by amplicon sequencing. This was probably due to the short SRT (2 d; 28°C),
411 resulting in washout of slower-growing microalgae (μ_{\max} 0.60-1.38 d⁻¹; 28°C).³⁷ It could,
412 therefore, be argued that SRT control and potentially additional measures in a real system are
413 crucial to prevent microalgal growth. Although the sulfate concentration in the medium was
414 1.2 g L⁻¹, there were no SRB detected (Figure 4) and no sulfate was removed (Supporting
415 Information S12). SRB require 0.7 g COD to remove 1 g of sulfate.⁴⁰ Therefore, they can
416 contribute to COD removal, yet biomass production will be negligible due to their low biomass
417 yields of 0.015-0.033 g VSS g⁻¹ SO₄²⁻.⁴¹ An anaerobic fermenter is proposed prior to the
418 raceway reactor, where sulfate can be converted to sulfide. The majority of PNSB grow only
419 at levels less than 45 mg S L⁻¹, yet some species of *Rhodobacter* and *Rhodoferax* can tolerate
420 sulfide concentrations up to 361 mg S L⁻¹.⁴² In practice, sulfide will not be problematic as
421 sulfate concentration, for example for brewery wastewater, are typical around 7 mg S L⁻¹.⁴³

422 It is unlikely that biological N₂ fixation by PNSB occurred, as nitrogen concentrations
423 were never limiting (effluent ammonium > 150 mg NH₄⁺ N L⁻¹). Interestingly, a gap of around
424 91-124 mg NH₄⁺ N L⁻¹ exists in the nitrogen balance (Supporting Information S12 Table S3).
425 This could in principle be due to nitrification and denitrification or through ammonia stripping.

426 Nitrification would theoretically be possible at an SRT of 2 days and a temperature of 28°C,
 427 yet no nitrifiers were detected in the microbial community (Figure 4) and the nitrite and nitrate
 428 concentration in the effluent was zero (Supporting Information S12 Table S3). At a pH of 7
 429 and a temperature of 28°C, 7% of the total ammonia nitrogen is available as free ammonia.
 430 CO₂ sparging for pH control and stirring of the paddlewheel may have contributed to NH₃
 431 stripping. Garcia, et al. 2000⁴⁴ for instance, observed that ammonia stripping accounted for 32-
 432 47% of the total nitrogen removal in an algae raceway reactor treating sewage (pH 8.6-9.4,
 433 temperature 12-27°C and HRT 3-10 d).



434 **Figure 3** Production features of a raceway reactor operated at a sludge retention time of 2 d, testing the effect of oxygen
 435 (stirring) and light (surface-to-volume ratio). Sampling occurred after the light (L) or dark (D) period. Results show (A) the
 436 protein productivity and the volumetric removal rate along with (B) the biomass yield and bacteriochlorophyll (BCA) content.
 437 Experiments were performed non-axenically with *Rhodobacter capsulatus* as initial inoculum. Stirring (on/off) 12h/12h
 438 implies stirring during the light period and not during the dark. Average values with standard error. Underlined text shows the
 439 change in reactor operation relative to the benchmark. Samples were taken at the end of the experiment. TSS: total suspended
 440 solids
 441



442

443 **Figure 4** Raceway reactor operated at a sludge retention time of 2 d, testing the effect of oxygen (stirring) and the combination
 444 of light (surface-to-volume ratio). Sampling occurred after the light (L) or dark (D) period. operational strategy on microbial
 445 community composition, Shannon's H' index, exp(H') and purple non-sulfur bacteria (PNSB) abundance. Stirring (on/off)
 446 12h/12h implies stirring during the light period and not during the dark. The PNSB genera *Rhodobacter* and
 447 *Rhodopseudomonas* are marked in orange. Underlined text shows the change in reactor operation relative to the benchmark.
 448 Samples were taken at the end of the experiment.

449 **3.4 Challenges for upscaling a raceway reactor for PNSB production on** 450 **wastewater**

451 This study used a synthetic medium, which contained only VFA as a carbon source. Acetate
 452 acid, propionic acid and butyric acid cannot be converted to secondary fermentation products
 453 such as butanol, hexanol and caproic acid without the presence of ethanol or hydrogen gas.⁴⁵
 454 In this experiment, the microbial competition was mainly between PNSB and aerobic
 455 chemoheterotrophs. Methanogens have also the potential to compete with PNSB for VFA, yet
 456 the short SRT of 2 d imposed on the system likely prevented their proliferation (minimal SRT
 457 methanogens 4-11 d at 28°C).⁶ Non-PNSB microbes might be additionally favored in real
 458 wastewater treatment due to its more complex organic composition and additional influent
 459 inflow of microbes, which may result in a lower proportion of PNSB in the microbial
 460 community. Pre-fermentation will convert a part of the COD. Due to the limited transfer of

461 microbes to the raceway reactor and residual fermentable COD, fermenters can grow and act
462 as symbionts by making COD available for PNSB.

463 Light limitation is a key challenge in a raceway reactor. It is mainly affected by the light
464 pass length and the suspended solids concentration.¹⁸ In this study, the reactor with a depth of
465 10 cm showed a productivity of 0.43 ± 0.03 g TSS L⁻¹ d⁻¹, which was 1.4 times higher compared
466 to one with a depth of 20 cm (Figure 3). The better performance for the system with a 10 cm
467 depth was due to the higher light availability per unit of biomass (0.64 W g⁻¹ TSS vs. 0.44 W
468 g⁻¹ TSS). More importantly for real wastewater, however, is the influence of incoming
469 suspended solids, which can cause light limitations to the system. Solid/liquid separation of the
470 incoming wastewater, for instance through a combination of coagulation/flocculation and
471 settling, is, therefore, still required to reduce turbidity and improve the light penetration into
472 the water.

473 Another operational challenge for realistic wastewater treatment is the pH control system.
474 In the current set-up, CO₂ sparging was used, as is practiced in industrial microalgae cultivation
475 in raceway reactors where CO₂ delivery is essential to avoid carbon limitations.⁴⁶ For PNSB,
476 CO₂ only serves as an electron sink for more reduced electron donors such as propionate and
477 butyrate.¹⁴ Acid dosage based on CO₂ sparging is, therefore, not essential for PNSB growth. In
478 the test set-up, the CO₂ sparging may unintentionally have created more favorable conditions
479 for PNSB by stripping out some dissolved oxygen (preventing additional aerobic conversions)
480 and sulfide (preventing potential toxicity). Follow-up research should also look into alternative
481 and economical pH control systems, for instance partially making use of the low influent pH
482 of fermentate.

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495

496 **Supporting Information.** Detailed methodology section 2.2.1; Influence of oxygen supply on
497 PNSB growth; Detailed methodology section 2.2.2; Light and oxygen as tool to steer PNSB
498 selectivity; Detailed methodology section 2.2.3; Illumination spectrum; Analytic procedures;
499 Detailed methodology microbial community analyses; Effect of SRT on COD removal rate;
500 Absorbance spectrum for raceway reactor; Dissolved oxygen concentration, nitrogen- and
501 sulfate balance and biomass parameters during raceway reactor operation. This information is
502 available free of charge via the Internet at

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