

1 **Control tools to selectively produce purple bacteria for microbial protein in raceway**  
2 **reactors**

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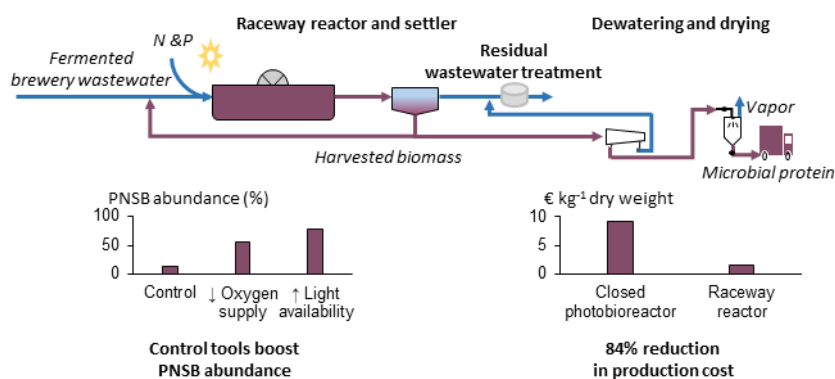
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15 **Graphical abstract**



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17

18 **Abstract**

19 Purple non-sulfur bacteria (PNSB) show potential for microbial protein production on  
20 wastewater as animal feed. They offer good selectivity (i.e. uneven community with high  
21 abundance of one species) when grown anaerobically in the light. However, the cost of a closed  
22 anaerobic photobioreactor (PBR) is prohibitive for protein production. While open raceway  
23 reactors are cheaper, their feasibility to selectively grow PNSB is thus far unexplored. This  
24 study developed tools to boost PNSB abundance in the biomass of a raceway reactor fed with  
25 volatile fatty acids as carbon source. For oxygen availability as tool, not stirring in the night  
26 (i.e. reduced oxygen supply) elevated the PNSB abundance from 8% to 20%. For light  
27 availability as tool, a 24-h illumination increased the PNSB abundance from 8% to 31%  
28 compared to a 12-h light/12-h dark regime. A reactor run at 2-d sludge retention time at the  
29 highest surface-to-volume ratio ( $10 \text{ m}^2 \text{ m}^{-3}$  increased light availability) showed productivities  
30 up to  $0.2 \text{ g protein L}^{-1} \text{ d}^{-1}$  and the highest PNSB abundance (78%). The estimated production  
31 cost is  $\text{€}1.9 \text{ kg}^{-1}$  dry weight (vs. PBR  $\text{€}11.4 \text{ kg}^{-1}$  dry weight). This study pioneered in PNSB-  
32 based microbial protein production in raceways, yielding cost efficiency along with high  
33 selectivity when avoiding the combined availability of oxygen, COD and darkness.

34

## 35 **1 Introduction**

36 Globally, only 4% of nitrogen and 17% of phosphorus fertilizers applied to the land, are  
37 eventually consumed.<sup>1,2</sup> These inefficiencies in the fertilizer-food chain severely distort the  
38 carrying capacity of the Earth, surpassing the planetary boundaries (i.e. safe operating space  
39 for sustainability) beyond the zone of uncertainty.<sup>3</sup> Mitigation can be brought about by  
40 upgrading wastewater resources to microbial protein or single-cell protein, which is the use of  
41 microorganisms as animal feed ingredient.<sup>4,5</sup> Resource recovery from food and beverage  
42 wastewater is preferred. Brewery wastewater is a key target as it offers a relatively high  
43 chemical oxygen demand (COD) concentration (800-9000 mg COD L<sup>-1</sup>) and easiness to  
44 prevent fecal contamination.<sup>6</sup>

45 Upgrading wastewater resources to microbial protein requires either chemo- or  
46 photoheterotrophic microorganisms to convert the organic carbon as well as non-axenic  
47 production conditions, as it is cost-wise redundant to sterilize vast amounts of water.<sup>4</sup>  
48 Chemoheterotrophs, also known as aerobic heterotrophic bacteria (AHB; i.e. aerobic activated  
49 sludge), make use of oxidation reactions for energy generation. These bacteria typically have  
50 low biomass yields (0.6 g COD<sub>biomass</sub> g<sup>-1</sup> COD<sub>removed</sub>) and high growth rates (2-6 d<sup>-1</sup>).<sup>7</sup> To date,  
51 AHB are pioneering in research, pilot, and full-scale implementations.<sup>8,9</sup> However, it is  
52 challenging to produce an AHB product characterized by an uneven microbial community with  
53 a high abundance of one dominant species (i.e. microbial selective production).<sup>8</sup>  
54 Photoheterotrophic cultivation of PNSB may offer such potential, because of their unique  
55 ability to grow highly selectively under anaerobic conditions in the light.<sup>10-12</sup> PNSB are  
56 characterized by high biomass yields (0.9-1.1 g COD<sub>biomass</sub> g<sup>-1</sup> COD<sub>removed</sub>) and have growth  
57 rates between 0.6-3.7 d<sup>-1</sup>.<sup>13-15</sup> However, compared to AHB, there is a lack of full-scale PNSB

58 facilities for microbial protein production, probably due to costs (expensive photobioreactor;  
59 PBR).

60 To achieve selectivity with PNSB (i.e. uneven microbial community and high abundance  
61 of one species), current research has focused on closed PBR such as anaerobic membrane  
62 bioreactors,<sup>16</sup> anaerobic tubular PBR<sup>13,17</sup> and illuminated anaerobic sequencing batch  
63 reactors<sup>18</sup>. These closed PBR only allow the growth of phototrophs and anaerobic chemotrophs.  
64 In the case of our previous study on synthetic wastewater, PNSB were able to be selectively  
65 produced with an abundance of *Rhodobacter capsulatus* between 93-97% and a low diversity  
66 index (exponent Shannon index) between 1.2-1.5 corresponding to an uneven microbial  
67 community.<sup>19</sup> A current cost estimation for PNSB-based protein production on wastewater in  
68 a closed PBR amounts to €22 kg<sup>-1</sup> protein,<sup>13</sup> which is roughly 11 times higher compared to the  
69 price of fishmeal of €2 kg<sup>-1</sup> protein.<sup>20</sup>

70 An economically more interesting case can be made if PNSB were produced in open  
71 raceway reactors conventionally used for microalgal processes.<sup>21</sup> These raceway reactors are  
72 open systems with a reactor depth around 20 cm (vs. 6 cm diameter tubular PBR), a surface-  
73 to-volume ratio of 5 m<sup>2</sup> m<sup>-3</sup> (vs. 22 m<sup>2</sup> m<sup>-3</sup> tubular PBR) and are agitated through a paddlewheel  
74 (vs. circulation pumps tubular PBR).<sup>21</sup> Investment costs of a raceway reactor approximate €56  
75 m<sup>-3</sup> compared to €5,000 m<sup>-3</sup> for a closed PBR.<sup>22,23</sup> However, achieving selective PNSB  
76 production is more challenging in these reactors, as air continuously enters the system, which  
77 enables the proliferation of competing aerobic heterotrophs (i.e. non-PNSB). Moreover, the  
78 oxygen concentration in a raceway reactor is zero due to its direct use as electron acceptor,  
79 making the growth of anaerobic chemotrophs also possible (e.g. acidogenic microorganisms  
80 and sulfate-reducing bacteria; SRB). Currently, there is no published research available on  
81 PNSB production with raceway reactors, except for a trial focusing on polyhydroxyalkanoate  
82 production.<sup>24</sup> However, it can be anticipated that the following control tools are essential to

83 maximize PNSB selectivity: (i) limiting the oxygen supply may decrease the growth of aerobic  
84 chemotrophs, (ii) increasing the light availability or the illumination period may aide PNSB in  
85 their competition for COD with aerobic chemotrophs and anaerobic chemotrophs such as  
86 acidogenic microorganisms and SRB, (iii) short sludge retention times (SRT) may washout  
87 slower-growing microorganisms such as microalgae, and (iv) limiting the COD-loading rate  
88 may decrease the competition with (an)aerobic chemotrophs during the dark period.

89 The hypothesis of this study was that PNSB can be selectively produced in a raceway  
90 reactor provided a good combination of control tools. Batch experiments were first performed  
91 to assess the phototrophic and (an)aerobic chemotrophic conversions of PNSB (axenic) and the  
92 (an)aerobic chemotrophic conversions of non-PNSB (non-axenic) to understand how they  
93 individually would contribute in a raceway reactor. Afterwards, the effect of oxygen supply,  
94 light availability, SRT and COD-loading rate was studied on PNSB abundance and community  
95 diversity (non-axenic). A raceway reactor was then operated at a SRT of 2 d to understand  
96 whether PNSB can be selectively produced over sequential batches and explore the best  
97 operational strategy for protein production and COD removal. All experiments were performed  
98 using a synthetic medium composed of volatile fatty acids (VFA). The findings of the raceway  
99 reactor were finally economically evaluated based on a production cost estimation, and  
100 benchmarked with the production of a PNSB biomass in a closed PBR.

## 101 **2 Materials and methods**

### 102 **2.1 Inocula and medium**

103 A *Rhodobacter capsulatus* strain, isolated in our previous study,<sup>13</sup> was used as model PNSB  
104 for the axenic flask and non-axenic raceway reactor experiments. This species was selected  
105 based on a prior evaluation made between five PNSB cultures, where it showed to have the  
106 highest photoheterotrophic growth rate on synthetic wastewater. This species is able to grow

107 photo- and chemoheterotrophically,<sup>10</sup> which enables examination of different PNSB growth  
108 kinetics in raceway reactors. Aerobic activated sludge of a local brewery company (AB InBev,  
109 Belgium, Leuven) was used as a proxy for a non-PNSB inoculum.

110 An adapted VFA-based medium as a proxy for fermented wastewater was used for all  
111 experiments, as we argued in a previous study that fermentation prior to protein production will  
112 favor the microbial selectivity.<sup>4,13</sup> The COD concentration was increased to 3 g L<sup>-1</sup> and  
113 contained a defined mixture of acetate, propionate and butyrate on a 1/1/1 COD basis. PNSB  
114 grown photoheterotrophically on this VFA mixture have a biomass yield that approximate 1 g  
115 COD<sub>biomass</sub> g<sup>-1</sup> COD<sub>removed</sub>.<sup>13,16</sup> This makes it easy to assess the chemoheterotrophic growth of  
116 PNSB and of competing non-PNSB, as a lower biomass yield implies oxidation of COD to  
117 CO<sub>2</sub>.

## 118 **2.2 Overview of the experiments**

119 Five sets of flask and raceway reactor experiments were performed in this study to explore the  
120 conversions of PNSB and the effect of oxygen supply, light availability and SRT on PNSB  
121 selectivity (Table 1).

122

123 **Table 1** Objectives and experimental setup of five tests to grow a protein-rich PNSB biomass on brewery wastewater. *Rhodobacter capsulatus* was used as purple non-sulfur bacterium (PNSB)  
 124 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  
 125 flasks were illuminated from the side and the raceway reactor from the top. OTR: oxygen transfer rate; SRT: sludge retention time

Objective	OTR (mg O <sub>2</sub> L <sup>-1</sup> d <sup>-1</sup> )	Stirring (on/off)	Illumination (light/dark)	S/V ratio (m <sup>2</sup> m <sup>-3</sup> )	SRT (d)	Inoculum	Cultivation	Reactor type
Assess conversions of PNSB and non-PNSB (section 2.2.1)	336	24 h / 0 h	24 h	61	Batch*	PNSB	Axenic	Flask
	0		24 h			PNSB		
	336		0 h			PNSB		
	0		0 h			PNSB		
	336		0 h			Non-PNSB	Non-axenic	
	0		0 h			Non-PNSB		
Influence of oxygen supply on PNSB growth (Supporting Information S2-3)	72	24 h / 0 h	24 h	61	Batch*	PNSB	Axenic	Flask
	336		24 h					
	72		0 h					
	336		0 h					
Oxygen and light availability as control tools to steer PNSB selectivity (section 2.2.2)	204	24 h / 0 h	12 h / 12 h	5	Batch*	PNSB	Non-axenic	Raceway
		24 h / 0 h	24 h / 0 h	5				
		12 h / 12 h	12 h / 12 h	5				
		24 h / 0 h	12 h / 12 h	10				
Effect of SRT on PNSB growth (section 2.2.3)	336	24 h / 0 h	12 h / 12 h	61	1.25	PNSB	Non-axenic	Flask
					2			
					3			
PNSB selectivity over sequential batches (section 2.2.4)	204	24 h / 0 h	12 h / 12 h	5	2	PNSB	Non-axenic	Raceway
		12 h / 12 h		5	2			
		24 h / 0 h		10	2			

126 \*Growth experiment between 50-150 h, stopped when stationary phase was reached

### 127 **2.2.1 Assess the conversions of PNSB and non-PNSB**

128 Flask batch experiments were performed to explore the photoheterotrophic and (an)aerobic  
129 chemoheterotrophic conversions of PNSB along with the (an)aerobic chemoheterotrophic  
130 conversions of competing non-PNSB. These tests were conducted to understand how these  
131 conversions may individually contribute in a raceway reactor. The detailed cultivation  
132 conditions are described in Supporting Information S1

133 To explore the conversions of PNSB, four different conditions were tested under axenic  
134 conditions: (i) illumination with oxygen supply to study the combined photo- and chemotrophic  
135 growth (conditions prevalent in a raceway reactor); (ii) illumination without oxygen supply to  
136 study the phototrophic growth; (iii) no illumination with oxygen supply to study the aerobic  
137 chemotrophic growth and (iv) no illumination without oxygen supply to study the anaerobic  
138 chemotrophic growth (i.e. acidogenic metabolism).

139 An experiment was also performed to assess the effect of oxygen supply on the photo-  
140 and chemoheterotrophic growth of PNSB. The methodology is explained in Supporting  
141 Information S2.

### 142 **2.2.2 Light and oxygen availability as control tools to steer PNSB selectivity**

143 These experiments were performed to explore the effects of light, oxygen supply, and the  
144 combination of light and oxygen on the biomass growth, biomass yield, biomass composition  
145 and PNSB selectivity.

146 A 100-L raceway reactor (MicroBio Engineering Inc., USA) was used to perform growth  
147 experiments in batch regime under non-axenic conditions. The stirring speed of the paddle was  
148 set at 30 rpm and the pH was controlled at 7 by sparging CO<sub>2</sub>. The maximum oxygen transfer  
149 rate (OTR) at this stirring speed was 204 mg O<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>. Temperature was controlled at 28°C  
150 with an external TetracHT 300-W heater (Tetra, Germany). Approximately 5 L of tap water  
151 was added daily to compensate for evaporation. One halogen lamp was used to illuminate the



152 raceway reactor at an intensity of  $54 \text{ W m}^{-2}$  (illumination spectrum see Supporting Information  
153 S4). The reactor was filled with the VFA-based medium and *Rhodobacter capsulatus* was used  
154 as inoculum at a total suspended solids (TSS) concentration of  $0.02 \text{ g L}^{-1}$ .

155 Four different conditions were tested in batch: (i) 12-h light/12-h dark with 24-h stirring  
156 at a surface-to-volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$  as benchmark (reactor filled up to 100 L); (ii) 24-h  
157 light/0-h dark with 24-h stirring at a surface-to-volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$  to study the effect of  
158 light; (iii) 12-h light/12-h dark with 12-h stirring (reduced oxygen supply vs. 24-h stirring)  
159 during the light period at a surface-to-volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$  to study the effect oxygen  
160 supply and (iv) 12-h light/12-h dark with 24-h stirring at a surface-to-volume ratio of  $10 \text{ m}^2 \text{ m}^{-3}$   
161 to study the effect of light (reactor filled up to 50 L). The absorbance of the biomass  
162 suspension (660 nm) was monitored to determine the maximum specific growth rate.  
163 Experiments were stopped when the stationary phase was reached. Samples were then taken  
164 for further analysis.

### 165 **2.2.3 Effect of SRT on PNSB growth**

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

168 Experiments were performed under combined photo- and chemotrophic conditions,  
169 allowing the entry of oxygen along with illumination (i.e. conditions prevalent in raceway  
170 reactor). Flasks of 500 mL were used as reactors and illuminated through a natural 12-h  
171 light/12-h dark regime with two halogen lamps at a light intensity of  $30 \text{ W m}^{-2}$  (vs. previous  
172 flask experiments section 2.2.1: 24-h light or 24-h dark). The flasks were filled with 200 mL  
173 of medium (section 2.1) corresponding to a maximum OTR of  $336 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$ . The  
174 experiment was performed non-axenically with *Rhodobacter capsulatus* as initial inoculum.  
175 The tested SRT were 1.25 d, 2 d and 3 d. All tests were performed in biological duplicate. After  
176 adding the medium and the inoculum, flasks were placed on a multipoint stirrer at 300 rpm.

177 Temperature during the light and dark period was respectively  $29.3 \pm 0.4^\circ\text{C}$  (i.e. resulting from  
178 radiation heat) and  $24.2 \pm 0.7^\circ\text{C}$ . The pH was  $7.4 \pm 0.2$ . Bottles were weighed daily to adjust  
179 for evaporation, remove a part of the broth and add fresh medium. Samples were taken daily  
180 to measure the absorbance (660 nm), pH, temperature and DO concentration. The moving  
181 average with a fixed subset size of three was determined. Steady state was reached when the  
182 daily absorbance overlapped with the moving average. Samples were then taken after the light  
183 and dark periods three days in a row to account for variability over time.

#### 184 **2.2.4 Operational strategies to steer PNSB selectivity and reactor performance**

185 A final experiment was performed to demonstrate that PNSB can be maintained in a raceway  
186 reactor over multiple generations and determine the best operational strategy in terms of  
187 productivity and COD removal.

188 Temperature, pH and stirring were controlled as described in section 2.2.2. Three  
189 operational strategies were tested: (i) 12-h light/12-h dark with 24-h stirring at a surface-to-  
190 volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$  as benchmark (reactor filled up to 100 L and depth 20 cm); (ii) 12-h  
191 light/12-h dark with 12-h stirring (reduces oxygen supply vs. 24-h stirring) during the light  
192 period at a surface-to-volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$  to study the effect oxygen supply and (iii) 12-h  
193 light/12-h dark with 24-h stirring at a surface-to-volume ratio of  $10 \text{ m}^2 \text{ m}^{-3}$  (higher light  
194 availability vs.  $5 \text{ m}^2 \text{ m}^{-3}$  reactor filled up to 50 L and depth 10 cm) to study the effect of light.  
195 The SRT was chosen based on the maximal specific growth rate during the batch experiments  
196 (Figure 2). A value of  $0.8 \text{ d}^{-1}$  was observed, which corresponds to a doubling time of 1.2 d. For  
197 safety reason, a SRT of 2 d was chosen for the three conditions to prevent washout from the  
198 reactor. Effluent was first removed and influent was then added before the start of the light  
199 period. The absorbance was analyzed daily. After steady state, samples were taken by the end  
200 of the light and dark periods three days in a row to account for variability over time. Samples  
201 were stored at  $-20^\circ\text{C}$  for further analysis.

### 202 **2.3 Analytical procedures**

203 Standard methods were used to determine the TSS and volatile suspended solids  
204 concentration.<sup>25</sup> The COD was measured using test kits (Macherey-Nagel, Germany) according  
205 to the manufacturer's instructions. The volumetric mass transfer coefficient ( $K_{La}$ ) of oxygen  
206 was determined according to the sulfite oxidation method.<sup>26</sup> Protein concentration was  
207 analyzed by Markwell, et al. 1978<sup>27</sup>, which is adapted from the Lowry procedure. The  
208 bacteriochlorophyll a content was determined by an acetone/methanol solvent (7:2 v/v).  
209 extraction.<sup>28</sup> Dissolved oxygen (DO) concentration (Hach, USA) and pH (Hanna Instruments,  
210 USA) were determined with Handheld meters.

### 211 **2.4 Microbial community analyses**

212 Genomic DNA was extracted from biomass samples collected (after steady state) across the  
213 reactor experiments using the DNeasy UltraClean microbial extraction kit (Qiagen, Venlo, the  
214 Netherlands) according to the manufacturer's instructions. The DNA extracts were sent to a  
215 commercial company (Novogene, China) for amplicon sequencing analysis. In brief, the V3-  
216 V4 hypervariable region of the bacterial 16S rRNA gene pool of the DNA extracts was  
217 amplified by PCR using the pair of 341f/806r primers prior to sequencing of PCR products  
218 using a HiSeq 2500 sequencer (Illumina, USA). A detailed description of the wet-lab and dry-  
219 lab workflows can be found in Supporting Information S5.

### 220 **2.5 Statistical analyses**

221 Statistical analyses were performed in R (version 3.4.1) using RStudio (RStudio®, USA) for  
222 Windows.<sup>29</sup> Student's t-test were conducted to compare means. Normality of data residuals  
223 was tested using the Shapiro-Wilk normality test. The assumption of homoscedasticity was  
224 verified through a Levene's test. The non-parametric Kruskal-Wallis rank sum test was  
225 executed when normality was rejected. The Welch's t-test was used in case of  
226 heteroscedasticity. A significance level of  $p < 0.05$  was chosen.

## 227 **2.6 Production cost estimation**

228 The cost of four PNSB production scenarios was estimated and compared: (i) a closed tubular  
229 PBR with 24-h stirring; (ii) a raceway reactor operated with 24-h stirring and a surface-to-  
230 volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$ ; (iii) a raceway reactor operated with 24-h stirring and a surface-to-  
231 volume ratio of  $10 \text{ m}^2 \text{ m}^{-3}$  and (iv) a raceway reactor operated with 24-h stirring during the  
232 light period and a surface-to-volume ratio of  $5 \text{ m}^2 \text{ m}^{-3}$ . Illumination for the four scenarios was  
233 considered to be from sunlight according to a natural 12-h light/12-h dark regime For the  
234 experiments, synthetic wastewater was used. This cost estimation was performed with brewery  
235 wastewater, as a suitable model for food and beverage effluents where fecal contamination can  
236 be avoided.

237 The goal of this model was to compare a closed PBR with a raceway reactor operated at  
238 three different strategies. It was not intended to determine an accurate production cost for  
239 PNSB. This cost estimation ought to be seen as a decision-making tool for research. Details  
240 and all input parameters are presented in Supporting Information S6.

## 241 **3 Results and discussion**

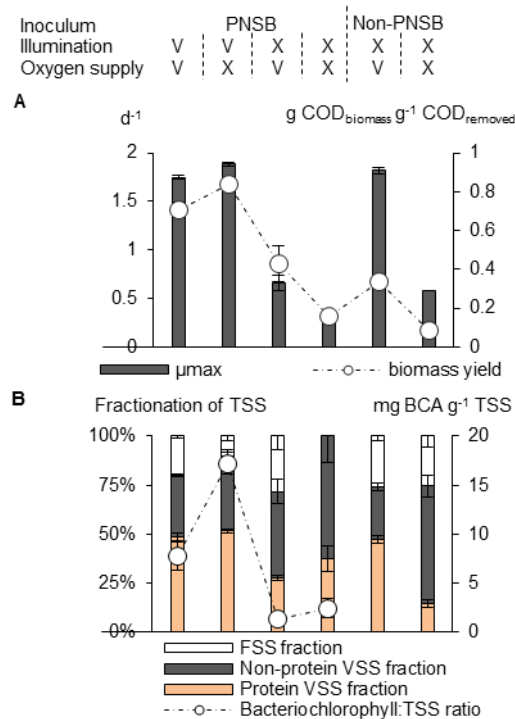
### 242 **3.1 Assess the conversions of PNSB and non-PNSB**

243 The results of the individual conversions of PNSB (Figure 1) indicated that during the  
244 combined photo- and chemotrophic growth (i.e. light and oxygen supply), the phototrophic  
245 metabolisms (i.e. under light) was dominant and not their chemotrophic metabolism (i.e.  
246 oxygen supply). Biomass yields for their phototrophic and the combined photo- and  
247 chemotrophic metabolisms were similar ( $p > 0.05$ ) and growth rates were almost equal. There  
248 was, therefore, more photo-assimilation of COD than oxidation to  $\text{CO}_2$ . The  
249 bacteriochlorophyll content was lower for the combined photo- and chemotrophic metabolism  
250 than for the phototrophic metabolism, yet still 6 times higher than for the aerobic chemotrophic

251 growth (i.e. no illumination with oxygen supply). It should be noted that the chemotrophic  
252 metabolism of PNSB contributes more to growth when the oxygen supply increases  
253 (Supporting Information S3).

254 A similar experiment was performed by Sasaki, et al. 1998<sup>30</sup> with *Rhodobacter*  
255 *sphaeroides* on orange peel waste, showing a 1.6 times higher biomass yield for the combined  
256 metabolism compared to the chemotrophic metabolism. Our results were in line with this  
257 finding. This reveals that PNSB have the potential to use both their photo- and chemotrophic  
258 metabolism at once.

259 The DO concentration in a raceway reactor is zero due to the direct consumption of  
260 oxygen, allowing anaerobic fermentation of COD. The anaerobic chemotrophic growth of  
261 PNSB was, therefore, tested with an organic more complex medium (vs. VFA-based medium  
262 see Supporting Information S1). PNSB were able to anaerobically ferment organics (Figure 1),  
263 yet growth rates and biomass yields were relatively low ( $0.3 \pm 0.08 \text{ d}^{-1}$ ;  $0.16 \pm 0.09 \text{ g COD}_{\text{biomass}}$   
264  $\text{g}^{-1} \text{ COD}_{\text{removed}}$ ). A similar observation was made by Schultz Weaver 1982<sup>31</sup> with low anaerobic  
265 growth rates and biomass yields for *Rhodobacter capsulatus* ( $\approx 0.08 \text{ d}^{-1}$ ;  $0.09 \text{ g COD}_{\text{biomass}} \text{ g}^{-1}$   
266  $\text{COD}_{\text{removed}}$ ) and *Rhodospirillum rubrum* ( $\approx 0.13 \text{ d}^{-1}$ ;  $0.11 \text{ g COD}_{\text{biomass}} \text{ g}^{-1} \text{ COD}_{\text{removed}}$ ). The  
267 non-PNSB inoculum showed growth rates of  $0.58 \pm 0.03 \text{ d}^{-1}$  or 2 times higher compared to  
268 PNSB. Therefore, anaerobic fermentation will mainly be performed by competing non-PNSB.  
269 Stronger competition during the light and dark period might arise from aerobic chemotrophic  
270 non-PNSB since their growth rate was 2.8 times higher than for the aerobic chemotrophic  
271 growth of PNSB and equal to the combined photo- and chemotrophic growth ( $p > 0.05$ ).



272

273 **Figure 1** (A) maximum specific growth rate and biomass yield for purple non-sulfur bacteria (PNSB) and non-PNSB along  
 274 with (B) biomass fractionation and bacteriochlorophyll a (BCA) content. Tested conditions: combined photo- and  
 275 chemoheterotrophic (illumination: V; oxygen supply: V), photoheterotrophic (V; X), aerobic chemoheterotrophic (X; V) and  
 276 anaerobic chemoheterotrophic (X; X) growth. The oxygen transfer rate was  $336 \text{ mg O}_2 \text{ L}^{-1} \text{ d}^{-1}$ . Experiments were performed  
 277 axenically with *Rhodobacter capsulatus* used as model PNSB. Non-PNSB were grown non-axenically. Averages with standard  
 278 error. TSS: total suspended solids; VSS: volatile suspended solids; FSS: fixed suspended solids i.e. ash

### 279 3.2 Light and oxygen availability as control tools to steer PNSB selectivity

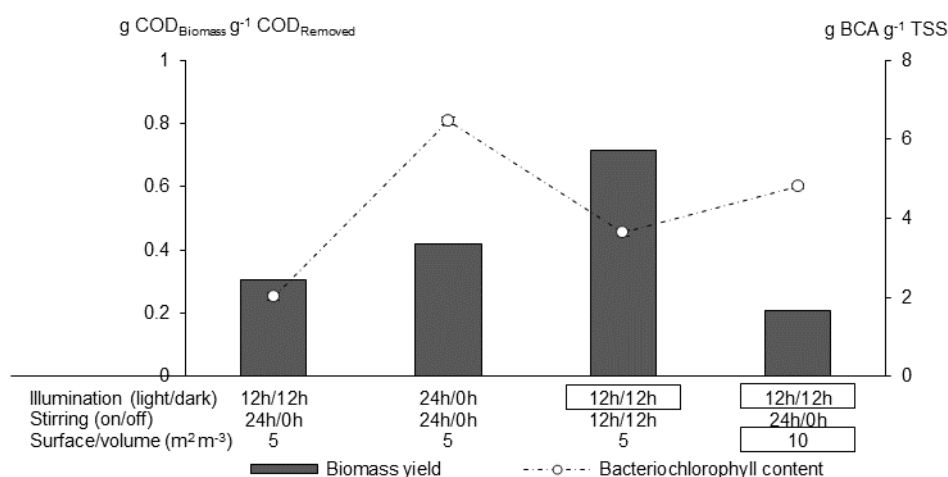
280 This experiment was set up to demonstrate that PNSB can be produced in a raceway reactor  
 281 under non-axenic conditions and to investigate the effect of light, oxygen supply and the  
 282 combination of both on PNSB selectivity.

283 The four tested conditions in Figure 2 showed an increase of the bacteriochlorophyll and  
 284 carotenoid peaks during the batch growth experiments. Microbial community analysis  
 285 confirmed these findings with PNSB abundances between 8-31% (Supporting Information S7).  
 286 Hence, this research pioneers in demonstrating PNSB production in a raceway reactor without  
 287 strict anaerobic conditions, thereby setting a precedent for future research. Lu, et al. 2019<sup>32</sup>

288 have also claimed to produce PNSB in a PBR at DO concentrations between 0.2-0.5 mg O<sub>2</sub> L<sup>-1</sup>,  
 289 yet have not presented results of the microbial community composition.

290 For light availability as control tool, increasing the illumination time to 24-h light/0-h  
 291 dark (vs. benchmark 12-h light/12-h dark) resulted in a biomass yield increase of 1.4 times  
 292 (more photo-assimilation), an increase of the bacteriochlorophyll content by 3.2 times and  
 293 increase of the PNSB abundance by 3.9 times (31% vs. benchmark 8%; Supporting Information  
 294 S7). This was also the most effective control tool in terms of PNSB abundance.

295 Preventing oxygen supply during the dark phase (not stirring vs. benchmark 24-h stirring)  
 296 increased the biomass yield by 2.3 times, the bacteriochlorophyll content by 1.8 times and the  
 297 PNSB abundance by 2.5 times (20%; Supporting Information S7). Hence, these results  
 298 reconfirm the findings of Supporting Information S3, where a lower oxygen supply resulted in  
 299 increased phototrophic growth. Increasing the surface-to-volume ratio had a dual effect.  
 300 Relatively to the benchmark, the biomass yield decreased by 1.5 times due to increased COD  
 301 oxidation and the bacteriochlorophyll content increased by 2.4 due to an increased light  
 302 availability per reactor volume.

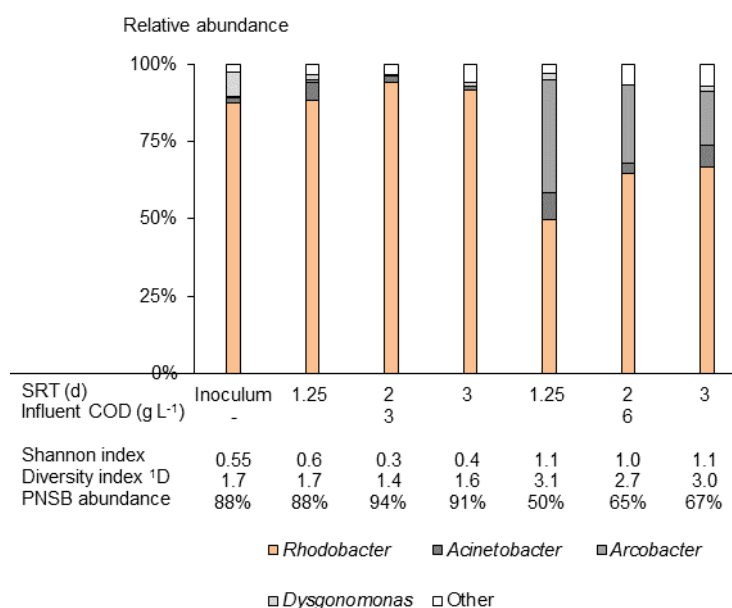


303  
 304 **Figure 2** Batch raceway reactor experiment testing the effects of light (illumination), oxygen (stirring) and the combination  
 305 of light (surface-to-volume ratio) on the biomass growth. Results show biomass yield (left y-axis) and bacteriochlorophyll a  
 306 (BCA) content (right y-axis). Experiments were performed non-axenically with *Rhodobacter capsulatus* as initial inoculum.

307 Stirring (on/off) 12 h / 12 h implies stirring during the light period and not during the dark. Rectangular boxes show the change  
 308 in reactor operation relative to the control. TSS: total suspended solids

### 309 3.3 Effect of SRT on PNSB growth

310 This experiment was performed to study the effect of SRT on PNSB selectivity. Overall, PNSB  
 311 abundances did not show substantial differences between SRT (Figure 3). This is in line with  
 312 our previous observation where we tested the effect of SRT on PNSB abundance in a closed  
 313 PBR.<sup>13</sup> Notably (Figure 3), the PNSB abundance at 6 g COD L<sup>-1</sup> for all SRT (50-67%) was  
 314 lower compared to 3 g COD L<sup>-1</sup> (88-94%). The exponent of the Shannon diversity index was  
 315 also lower at 3 g COD L<sup>-1</sup> (1.4-1.7) compared to 6 g COD L<sup>-1</sup> (2.7-3.0), which indicates a more  
 316 uneven community at lower loading rates. The abundance of the (an)aerobic  
 317 chemoheterotrophs *Arcobacter* was predominantly higher at higher loading rates (17-36%).  
 318 Overall, it can be concluded that a higher loading decreased the PNSB selectivity. This  
 319 decreased PNSB selectivity was probably due to the higher COD availability especially during  
 320 the night period, leading to increased growth of competing chemotrophs. As such, COD  
 321 availability during the night will negatively influence the PNSB abundance.



322



323 **Figure 3** Effect of sludge retention time (SRT) on microbial community composition, Shannon's H index,  $\exp(H')$  and purple  
324 non-sulfur bacteria (PNSB) abundance. Flasks were used as a reactor. The PNSB genera *Rhodobacter* and *Rhodopseudomonas*  
325 are all marked in orange. Samples obtained after 2-10 SRT.

### 326 **3.4 Reactor performance and community dynamics over sequential batches**

327 This experiment was conducted to demonstrate that PNSB can be maintained in a raceway  
328 reactor over multiple generations and determine the best operational strategy in terms of  
329 productivity and COD removal.

330 The highest productivities ( $0.21 \text{ g protein L}^{-1} \text{ d}^{-1}$  corresponding with  $0.43 \text{ g TSS L}^{-1} \text{ d}^{-1}$ )  
331 and removal rates ( $0.79 \text{ g COD L}^{-1} \text{ d}^{-1}$ ) were achieved when the reactor was operated with 24-  
332 h stirring and 12-h light/12-h dark at the highest surface-to-volume ratio of  $10 \text{ m}^2 \text{ m}^{-3}$  (Figure  
333 4). A higher ratio of  $10 \text{ m}^2 \text{ m}^{-3}$  increased the light availability, resulting in higher biomass  
334 concentrations ( $0.81 \pm 0.04 \text{ g TSS L}^{-1}$ ) relative to the benchmark of  $5 \text{ m}^2 \text{ m}^{-3}$  ( $0.62 \pm 0.02 \text{ g}$   
335  $\text{TSS L}^{-1}$ ). For a closed PBR operated on the same medium at a SRT of 1 d (vs. 2 d for raceway  
336 reactor), we reached TSS productivities that were 1.5-2.6 times higher compared to the raceway  
337 reactor.<sup>13</sup> This was probably due to the higher light availability in the PBR compared to a  
338 raceway reactor (surface-to-volume ratio 33 vs. 5-10  $\text{m}^2 \text{ m}^{-3}$ ). For the microalga *Chlorella*  
339 *vulgaris* cultivated in the same reactor (12-h light/12-h dark), the productivity was  $0.009 \text{ g}$   
340  $\text{protein L}^{-1} \text{ d}^{-1}$  or 22 times lower compared to PNSB.<sup>6</sup> This was probably due to the higher  
341 growth rates of PNSB of  $0.6\text{-}3.7 \text{ d}^{-1}$  compared to the ones of microalgae of  $0.60\text{-}1.38 \text{ d}^{-1}$ .<sup>13,33,34</sup>  
342 To a lesser extent, non-PNSB chemotrophs also contributed to biomass production in the  
343 raceway reactor, thereby increasing the overall biomass productivity.

344 In terms of PNSB selectivity (Figure 5), preventing the combination of oxygen supply  
345 and darkness (not stirring) was an effective strategy in line with the results of section 3.2. The  
346 PNSB abundance was 56% (vs. 14% benchmark 24-h stirring) and the microbial community  
347 was more uneven showing a lower exponent of the Shannon diversity index (3.7 vs. 4.3 for  
348 benchmark). The decrease in PNSB abundance to 41% was notable during the dark period

349 along with the increase of the exponent of the Shannon diversity index from 3.7 to 7.2. Hence,  
350 biomass harvesting should preferably be performed after the light period to assure a selective  
351 microbial community.

352 The reactor operated with 24-h stirring at a surface-to-volume ratio of  $10 \text{ m}^2 \text{ m}^{-3}$  was the  
353 best strategy in terms of PNSB selectivity, showing a PNSB abundance of 78% or 5.6 times  
354 higher compared to the benchmark and very comparable to the inoculum. The exponent of the  
355 Shannon diversity index was only 2.5, the lowest for all conditions and even lower than the  
356 inoculum (3.5). This implies that light availability is key to boost PNSB selectivity in a raceway  
357 reactor. The findings also show that a raceway reactor can approach the PNSB selectivity of a  
358 closed PBR. Potential higher PNSB abundances might even be achieved if oxygen supply is  
359 prevented during the night along with high surface-to-volume ratios.

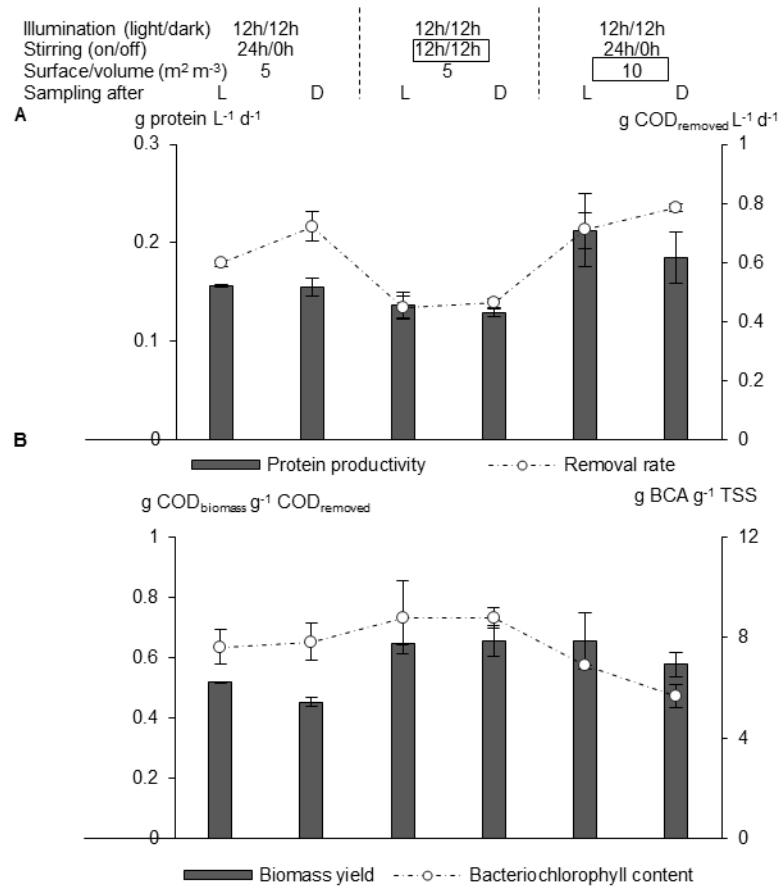
360 Other PNSB genera were also present in the system such as *Rhodopseudomonas* (2-3%),  
361 and *Blastochloris* (< 0.2%). The main competing genera were *Acinetobacter* (aerobic  
362 chemoheterotroph), *Dysgonomonas* (anaerobic chemoheterotrophs), *Arcobacter* ((an)aerobic  
363 chemoheterotrophs) and *Alcaligenes* (aerobic chemoheterotrophs) with an abundance of  
364 respectively 1-4%, 1-12%, 0-31% and 1-54%. Microalgae were not detected through the  
365 absorbance spectra (no chlorophyll peaks) and no cyanobacteria were identified by amplicon  
366 sequencing. This was probably due to the short SRT (2 d), resulting in washout of slower-  
367 growing microalgae ( $\mu_{\text{max}}$  0.60-1.38  $\text{d}^{-1}$ ).<sup>33</sup> Although the sulfate concentration in the medium  
368 was  $1.6 \text{ g L}^{-1}$ , there were no SRB detected (of note, primers not designed for archaea). SRB  
369 require 0.7 g COD to remove 1 g of sulfate.<sup>35</sup> Therefore, they can contribute to COD removal,  
370 yet biomass production will be negligible due to their low biomass yields of 0.015-0.033 g VSS  
371  $\text{g}^{-1} \text{ SO}_4^{2-}$ .<sup>36</sup>

372 According to the authors' knowledge, this study is the first to publish results on PNSB  
373 production in a raceway reactor dedicated to microbial protein. Literature studying microbial

374 communities in waste lagoons are helpful for benchmarking, as these systems enable  
375 photosynthesis and are open to air. Do, et al. 2003<sup>37</sup> have investigated the correlation of  
376 environmental factors on photosynthetic blooms, i.e. the spontaneous growth of purple bacteria  
377 in waste lagoons. Their research showed that for a swine waste lagoon, up to 10% of the  
378 microbial community was made by a population of *Rhodobacter*. The authors observed a  
379 positive correlation between *Rhodobacter* and the sulfate concentration. They claimed that it  
380 was due to competition between PNSB and SRB. More PNSB growth would result in a lack of  
381 VFA for SRB and the accumulation of sulfate.

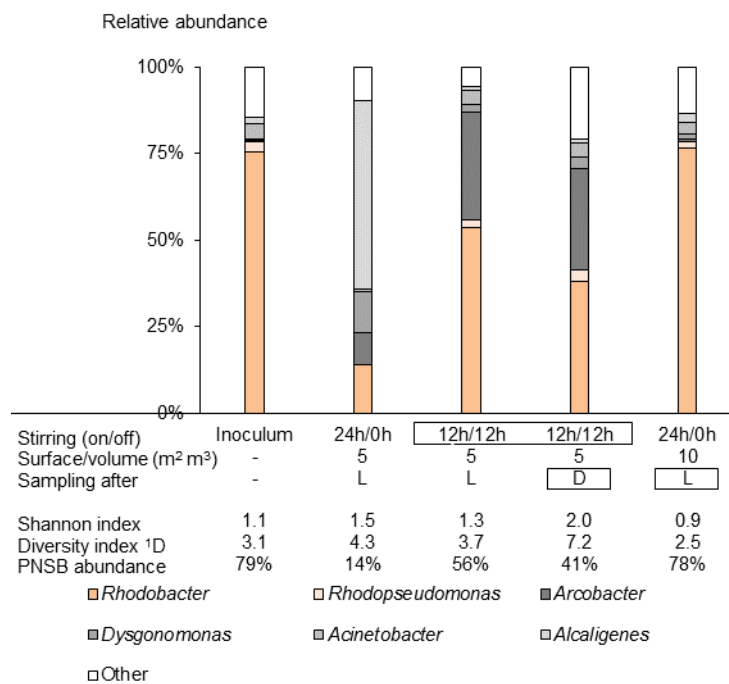
382 Our reactor was operated with artificial 12h-light/12h-dark conditions at constant  
383 temperature with a VFA-based medium. Hence, sunlight-driven pilot-scale validation should  
384 still confirm these findings. As scale-up for raceways is mainly horizontal (depth is kept  
385 constant), the main effects are expected from a different influent composition (more complex  
386 COD mixture vs. synthetic), fluctuations in COD flow and temperature, day/night length and  
387 light intensity. Another parameter that might be different for a for a full-scale raceway reactor  
388 is the oxygen supply to the system. The oxygen supply rate during the experiment in this  
389 chapter was around 204 mg O<sub>2</sub> L<sup>-1</sup> d<sup>-1</sup>. However, for a full-scale system this would potentially  
390 be lower due to higher reactor volume. The lower oxygen supply would positively influence  
391 the PNSB growth, yet might enhance the competition with anaerobic heterotrophs such as SRB  
392 and fermentative microorganisms.

393



394

395 **Figure 4** Production features of a raceway reactor operated at a sludge retention time of 2 d, testing the effect of oxygen  
 396 (stirring) and light (surface-to-volume ratio). Sampling occurred after the light (L) or dark (D) period. Results show (A) the  
 397 protein productivity and the volumetric removal rate along with (B) the biomass yield and bacteriochlorophyll (BCA) content.  
 398 Experiments were performed non-axenically with *Rhodobacter capsulatus* as initial inoculum. Stirring (on/off) 12 h / 12 h  
 399 implies stirring during the light period and not during the dark. Average values with standard error. 12 h / 0 h stirring occurred  
 400 during the light period. Rectangular boxes show the change in reactor operation relative to the benchmark. TSS: total suspended  
 401 solids



402

403 **Figure 5** Effect of operational strategy of a raceway reactor on microbial community composition, Shannon's H' index,  
 404 exp(H') and purple non-sulfur bacteria (PNSB) abundance. Raceway reactor operated at a sludge retention time of 2 d, testing  
 405 the effect of oxygen (stirring) and the combination of light (surface-to-volume ratio). Sampling occurred after the light (L) or  
 406 dark (D) period. Stirring (on/off) 12 h / 12 h implies stirring during the light period and not during the dark. The PNSB genera  
 407 *Rhodobacter* and *Rhodopseudomonas* are marked in orange. Rectangular boxes show the change in reactor operation relative  
 408 to the benchmark.

### 409 3.5 Production cost comparison of a closed PBR and an open raceway reactor

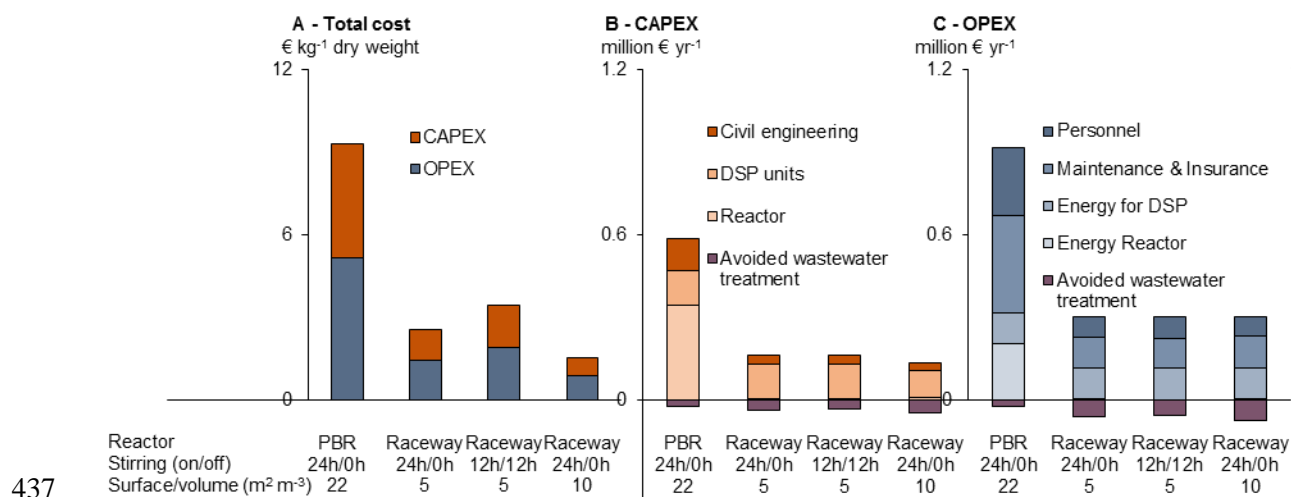
410 An anaerobic reactor such as a closed PBR is the best non-axenic technology to selectively  
 411 produce PNSB on wastewater, yet production costs were estimated between 2.7-6.4 times  
 412 higher compared to a raceway reactor (Figure 6). These high costs can mainly be contributed  
 413 to the high investment costs for a tubular PBR (47% of capital expenditure; CAPEX) and the  
 414 energy required for recirculation (23% of operational expenditure; OPEX).

415 PNSB cultivated in closed PBR have high biomass yields (1 g COD<sub>biomass</sub> g<sup>-1</sup> COD<sub>removed</sub>)  
 416 due to photo-assimilation instead of oxidation of COD to CO<sub>2</sub>. This will result in lower COD  
 417 removal efficiencies compared to a raceway reactor. A raceway reactor can save around  
 418 €83,000-120,000 yr<sup>-1</sup> of the wastewater treatment cost or 1.7-2.4 times more compared to a

419 PBR. Raceway reactors have the potential to remove 88% of the incoming COD. Therefore,  
 420 wastewater treatment after PNSB production with a raceway reactor can be a simple aerobic  
 421 activated sludge treatment process instead of a digester followed by aerobic treatment. On the  
 422 contrary, PBR only remove 35% of the COD. Hence, a digester is still needed to lower the  
 423 COD concentration.

424 The cost to produce PNSB in a raceway reactor was estimated between €1.3-3.0 kg<sup>-1</sup> dry  
 425 weight (DW). Downstream processing was the most dominant cost factor contribution by 57-  
 426 61% to the CAPEX and by 40-43% to the OPEX. A further cost reduction of 27-40% can be  
 427 possible if ultrafiltration is replaced by gravitational settling (€0.9-2.4 kg<sup>-1</sup> DW). Currently,  
 428 there is no published literature studying the settleability of PNSB except for Cerruti, et al. 2020  
 429 DOI: <https://doi.org/10.1101/2020.01.08.899062><sup>38</sup>. Further research should explore this  
 430 closely, as it will be an important cost saver.

431 The operational strategy with 24-h stirring at a surface-to-volume ratio of 10 m<sup>2</sup> m<sup>-3</sup>  
 432 (reactor depth 10 cm) resulted in the highest PNSB selectivity and was also the most cost-  
 433 effective. A regime of 24-h stirring will produce more biomass (more chemotrophic growth)  
 434 and will remove more COD relatively to 12-h stirring. This will result in a cost that can be  
 435 spread out over more products and additional savings for the subsequent aerobic activated  
 436 sludge process.



438 **Figure 6** Cost comparison for a closed tubular photobioreactor (PBR) and an open raceway reactors using natural sunlight  
439 (12-h light/12-h dark). Results show (A) capital and operational expenditure (CAPEX, OPEX), (B) fractionation of CAPEX,  
440 and (C) fractionation of OPEX. The cost category “Avoided wastewater treatment” refers to activated sludge treatment costs  
441 that can be avoided due to COD removal by the microbial protein reactor. Stirring (on/off) 12 h / 12 h implies stirring during  
442 the light period and not during the dark. All scenarios were dimensioned based on brewery wastewater with a COD  
443 concentration of 2.1 kg m<sup>-3</sup> and a flow rate of 1150 m<sup>3</sup> d<sup>-1</sup>. DSP: Downstream processing i.e. harvesting, dewatering and drying.

## 444 **4 Conclusions**

445 The goal of this research was to develop control tools to selectively produce PNSB in a raceway  
446 reactor. The main findings of this study are:

- 447 (i) This study pioneers in the selective production of PNSB in a raceway reactor with  
448 productivities of up to 0.43 g TSS L<sup>-1</sup> d<sup>-1</sup> and COD removal rates of up to 0.79 g COD  
449 L<sup>-1</sup> d<sup>-1</sup>.
- 450 (ii) Avoiding oxygen supply during the dark phase and a higher surface-to-volume ratio  
451 were the best operational strategies to maximize the PNSB abundance (56-78%) and  
452 lower the diversity.
- 453 (iii) SRT does not show to have an impact on PNSB selectivity. However, COD availability  
454 should be avoided in the dark, as it decreases the PNSB abundance from 90% to 69%  
455 and increases the Shannon diversity from 0.45 to 1.1.
- 456 (iv) Flask and raceway experiments showed that PNSB competed mainly with aerobic  
457 chemotrophs and to a minor extent with anaerobic chemotrophs. Microalgae or SRB  
458 were not identified as major competitors.
- 459 (v) The combination of oxygen supply, higher COD load and darkness should be avoided.
- 460 (vi) Production costs for a raceway reactor amount to €1.9 kg<sup>-1</sup> DW, which is six times  
461 cheaper than a closed PBR.

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472



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