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Cocultivating aerobic heterotrophs and purple bacteria for microbial protein in sequential photo- and chemotrophic reactors

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1 **Cocultivating aerobic heterotrophs and purple bacteria for microbial protein in**

2 **sequential photo- and chemotrophic reactors**

3

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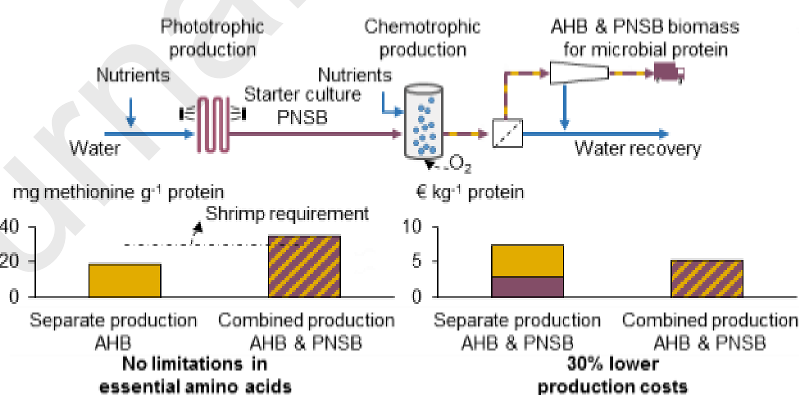
14 ## Graphical abstract



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16 ## Abstract

17 Aerobic heterotrophic bacteria (AHB) and purple non-sulfur bacteria (PNSB) are typically
18 explored as two separate types of microbial protein, yet their properties as respectively a bulk
19 and added-value feed ingredient make them appealing for combined use. The feasibility of
20 cocultivation in a sequential photo- and chemotrophic approach was investigated. First,
21 mapping the chemotrophic growth kinetics for four *Rhodobacter, Rhodopseudomonas and*
22 *Rhodospirillum* species on different carbon sources showed a preference for fructose (μ_{max}
23 2.4-3.9 d^{-1} 28°C; protein 36-59%$_{DW}$). Secondly, a continuous photobioreactor inoculated with
24 *Rhodobacter capsulatus* (VFA as C-source) delivered the starter culture for an aerobic batch
25 reactor (fructose as C-source). This two-stage system showed an improved nutritional quality
26 compared to AHB production: higher protein content (45-71%$_{DW}$), more attractive
27 amino/fatty acid profile and contained up to 10% PNSB. The findings strengthen protein
28 production with cocultures and might enable the implementation of the technology for
29 resource recovery on streams such as wastewater.

30

31 **Keywords:** purple phototrophic bacteria; single-cell protein; alternative protein; animal feed;
32 aquafeeds

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33 # 1 Introduction

34 A key challenge during the Anthropocene is to increase high-quality food production while
35 mitigating climate change, the distortion of the biochemical nitrogen and phosphorus flows,
36 biodiversity loss, freshwater use and land use (Pikaar et al., 2017; Steffen et al., 2015).
37 Alternative fertilizer-to-food systems are essential (Verstraete et al., 2016), as the
38 conventional food chain suffers from nutrient losses such as leaching, runoff and
39 volatilization (Galloway et al., 2003). Lowering agriculture crop production through direct
40 use of nutrients for the production of microbial biomass as a source of animal feed has the
41 potential to increase the overall nitrogen efficiency from 4 to 10% (Pikaar et al., 2017).

42 This microbial biomass, so-called microbial protein (i.e. single-cell protein), can be
43 produced with various types of yeast, fungi, algae and bacteria (Matassa et al., 2016). The
44 production is typically performed with synthetic media from primary or renewable origin or
45 on waste streams such as wastewater for resource recovery (Najafpour, 2015; Verstraete et
46 al., 2016). Microbial protein production on synthetic media is mainly dominated by axenic
47 fermenter technology, which enables culture specificity (Najafpour, 2015). On the other
48 hand, the production of microbial protein for resource recovery is usually performed with
49 non-axenic heterotropic cultures such as aerobic heterotrophic bacteria (AHB), purple non-
50 sulfur bacteria (PNSB) and consortia of microalgae and AHB (Spiller et al., 2020).

51 AHB cultivation is the production of a consortium of bacteria under aerobic
52 chemoheterotrophic conditions on wastewater (Vriens et al., 1989). These microbes have a
53 high protein content (38-60 g protein 100 g^{-1} total suspended solids; TSS), an appealing
54 essential amino acid (EAA) profile and contain several vitamins (e.g. B1, B2, B6, B12)
55 (Vriens et al., 1989). They are mostly studied as a bulk feed ingredient, yet some studies
56 indicate potential beneficial effects against pathogenic bacteria in aquaculture and prebiotic

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57 potential due to the presence of poly-β-hydroxybutyrate in their biomass (Crab et al., 2012).
58 PNSB are gram-negative microbes and belong to the purple bacteria, which also comprise the
59 purple sulfur bacteria (Blankenship et al., 1995). Purple bacteria should not be confused with
60 the microbiological term of 'purple' for gram-positive bacteria in Gram staining. Contrary to
61 AHB, PNSB are mainly explored in anaerobic photobioreactors (PBR) for their
62 photoheterotrophic metabolism (Capson-Tojo et al., 2020). They have been studied for axenic
63 cultivation on synthetic media, yet more recent literature focusses on wastewater with non-
64 axenic cultures (Capson-Tojo et al., 2020). The main difference between PNSB and AHB is
65 the possibility of the former for microbial selective production when cultivated under
66 anaerobic photoheterotrophic conditions (i.e. uneven community with a high abundance of
67 one species; (Alloul et al., 2019; Cerruti et al., 2020; Hülsen et al., 2016a; Hülsen et al.,
68 2016b). Production of PNSB, is, however, more expensive than for AHB. Investment costs of
69 a closed anaerobic PBR approximate € 5,000 m^{-3} compared to € 300 m^{-3} for an aerobic tank
70 (Acien et al., 2012; van Haandel & van der Lubbe, 2012). Moreover, PNSB growth is limited
71 by light availability for the cells, which results in lower biomass concentrations and
72 consequently lower biomass productivities such as 4.2 g COD L^{-1} d^{-1} for photo-anaerobic
73 membrane bioreactors (Capson-Tojo et al., 2020) compared to AHB (oxygen transfer is rate
74 limiting, not light). The biomass of PNSB is appealing with a high protein content (40-61 g
75 protein 100 g^{-1} TSS), an outstanding protein quality (appealing profile EAA) and vitamins
76 such as B1, B2, B3, B5, B6, B9, E and biotin (Sasaki et al., 1998). PNSB are studied as a
77 feed ingredient, yet they are unique due to their added-value properties beyond the nutritional
78 content: (i) they enhance the growth performance of several fish species and shrimp (Alloul
79 et al., 2021; Chowdhury et al., 2016; Delamare-Deboutteville et al., 2019; Noparatnaraporn et
80 al., 1987; Shapawi et al., 2012), (ii) have antimicrobial properties against shrimp *Vibrio*
81 pathogens as demonstrated by our previous work (Alloul et al., 2021), (iii) contain

82 antioxidants such as carotenoids (Sasaki et al., 1998) and (iv) can have color benefits for
83 aquaculture animals (Noparatnaraporn et al., 1987). These bacteria can also serve as an
84 astronaut food ingredient in regenerative life-support systems (Clauwaert et al., 2017) and
85 live or dried PNSB have added value in crop production (Spanoghe et al., 2020).

86 AHB and PNSB are, currently, explored as two separate types of microbial protein, yet
87 their properties as respectively a bulk and added-value protein ingredient make them
88 appealing for combined use. A community containing a relatively high proportion of AHB
89 and a relatively low proportion of PNSB might be an interesting balance between high
90 production costs of PNSB and their addition of added-value properties to the product.
91 Obtaining a combined product is possible by producing both types of microbes in separate
92 reactors followed by blending. However, PNSB are also able to grow aerobic
93 chemotrophically, which, thus, in principle enables cocultivation with AHB, provided that the
94 reactor configuration and operational conditions prevent overgrowth of one culture by the
95 other.

96 This study proposes a 'hybrid' non-axenic photo- and chemotrophic production system.
97 PNSB are first pre-cultivated phototrophically on synthetic medium to offer them a
98 competitive advantage in the subsequent chemotrophic production step. Such a system
99 requires insights in the photo- and chemotrophic PNSB growth kinetics, yet extensive
100 knowledge of their chemotrophic growth characteristics is lacking. Several researchers have
101 focused on axenic chemotrophic growth of pure PNSB species exploring the pigment
102 formation during the dark and the expression of special compounds such as ubiquinone (Yen
103 & Chiu, 2007; Zeiger & Grammel, 2010). Comparative screening of the chemotrophic
104 growth kinetics of different PNSB species on different carbon sources is limited to
105 *Rhodospirillum rubrum* (growth rate 3.0-3.1 d^{-1}) and *Rhodobacter capsulatus* on succinate,
106 fructose and acetate (Schultz & Weaver, 1982; Zeiger & Grammel, 2010). An investigation

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107 of the community structure and performance of AHB seeded with phototrophic PNSB has not
108 been explored so far.

109 This research aims to investigate the feasibility of the 'hybrid' system for the
110 cocultivation of AHB and PNSB as a combined source of microbial protein. The first
111 objective of this study was to select the most suitable PNSB inoculum, by comparing the
112 chemotrophic growth kinetics of *Rhodobacter capsulatus*, *Rb. sphaeroides*,
113 *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* on three carbon types: volatile
114 fatty acids (VFA), alcohols, and carbohydrates. Apart from growth kinetics, the metabolic
115 flexibility to switch from photo- to chemotrophic growth, protein content and biomass yield
116 under chemotrophic conditions were used as performance metrics as well. The second goal
117 was to explore (and optimize) a two-stage photo- and chemotrophic reactor system. The best
118 PNSB from the batch tests was used as inoculum in a non-axenic semi-continuous PBR
119 coupled to an aerobic reactor operated in batch. Effects of dissolved oxygen (DO)
120 concentration and addition of an AHB inoculum were studied in terms of productivity,
121 nutritional quality (protein content, essential amino and fatty acid content) and microbial
122 community structure of the AHB & PNSB consortium.

123 2 Materials and methods

124 2.1 PNSB species

125 To screen for the best PNSB culture for a two-stage photo- and chemotrophic production
126 system, six cultures were pre-selected. Four pure cultures were chosen, namely *Rb.*
127 *capsulatus, Rb. sphaeroides* LMG 2827, *Rhodopseudomonas palustris* LMG 18881 and
128 *Rhodospirillum rubrum* S1H. These species were chosen because they are one of the most
129 studied PNSB, enabling a benchmark to previous literature (Capson-Tojo et al., 2020).

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130 The last two selected cultures were a 3-species synthetic community (i+ii+iii) to study
131 potential synergistic effects and an AHB inoculum originated from aerobic return sludge of a
132 local brewery company (AB InBev, Belgium, Leuven). Axenic PNSB cultures were pre-
133 cultivated under anaerobic phototrophic conditions with a pre-autoclaved VFA-based
134 medium adapted from Alloul et al. (2019). The AHB inoculum was chemotrophically pre-
135 cultivated in the same medium.

136 **2.2 Chemotrophic growth kinetics and yield in batch incubations**

137 Chemotrophic batch tests were divided into two experimental setups: (i) a preliminary
138 screening was performed with nine different carbon sources in 96-Well plates and (ii) four
139 carbon sources were selected for the second experiment in Erlenmeyer flasks based on the
140 growth kinetics in the 96-Well plates.

141 The 96-Well plate experiments were performed in a working volume of 150 μL. The
142 medium of Alloul et al. (2019) was used and the VFA were replaced by another carbon
143 source (chemical oxygen demand basis; COD) depending on the experiment. A total of nine
144 carbon sources were tested in triplicate containing four VFA typically used to cultivate PNSB
145 (acetate, propionate, butyrate and a VFA mixture 1/1/1 ratio on COD basis), three
146 carbohydrates (fructose, glucose and sucrose) and two alcohols (glycerol and ethanol) at a
147 COD concentration of 3 g L^{-1}. In this medium, the KH_2PO_4 content was adapted to 2.7 g-P L^{-}
148 ^{1} to cope with pH increase. The pH of the media was adjusted to 7.0 before the experiment by
149 adding 12 M of NaOH and autoclaved (reducing sugars added after autoclaving). *Rb.*
150 *capsulatus, Rb. sphaeroides, Rps. palustris* and *Rsp. rubrum* were first phototrophically pre-
151 cultivated and then supplemented to the wells at an initial optical density of 0.200 (OD_{660nm}).
152 Plates were then incubated in a microplate plate reader (Biotek, USA) at 28°C with vigorous
153 orbital shaking (282 rpm) for aeration. The growth was monitored by measuring the OD_{660nm}
154 every 2.5 h.

155 After the 96-Well plate pre-screening, four different carbon sources were selected, and
156 experiments were repeated in 500 mL Erlenmeyer flasks with a working volume of 200 mL.
157 All six cultures described in section 2.1 were tested in triplicate. The pH of the media was
158 adjusted to 7.0 before autoclaving. The flasks were then inoculated at an initial concentration
159 of 0.03 g TSS L^{-1}. Experiments were performed in a climate chamber (Snijders Scientific,
160 The Netherlands) at 28°C. Flasks were covered with aluminum foil to prevent light
161 penetration and placed on a multipoint stirrer at 300 rpm (Thermo Scientific, USA) for
162 aeration (kLA 2 h^{-1}). The growth was monitored by measuring the absorbance at 660 nm.

163 2.3 Two-stage photo- and chemotrophic reactor setup and operation

164 The sequential system consisted of a non-axenic semi-continuous anaerobic PBR coupled to
165 an aerobic reactor operated in batch. The main goal of the PBR was to offer PNSB a
166 competitive advantage in the aerobic reactor.

167 2.3.1 Phototrophic production in a closed photobioreactor

168 The non-axenic PBR was a vertical tubular vessel with a working volume of 500 mL and an
169 external diameter of 6 cm (surface to volume ratio 67 m^2 m^{-3}). It was operated at an SRT of
170 0.93 ± 0.1 d for 87 days at a temperature of 28 ± 2 °C, a volume exchange ratio of 54 ± 4%,
171 illuminated with two halogen lamps at a light intensity of 30 W m^{-2} and stirred with a
172 magnetic stirrer at 700 rpm (Carl Roth, Germany). The reactor was operated semi-
173 continuously, by removing 250 mL effluent and adding 250 mL influent every 12h. The gas
174 outlet was connected to a nitrogen gas expansion balloon to cope with under- and
175 overpressure during withdrawal and fill. The reactor was inoculated with *Rb. capsulatus*,
176 which was shown to be the most promising PNSB based on the chemotrophic batch
177 experiments. VFA were chosen as they are the preferred carbon source for the
178 photoheterotrophic growth of PNSB (Blankenship et al., 1995). A VFA mixture adapted from
179 Alloul et al. (2019) was used at a 1/1/1 ratio on COD basis: 1 g acetic acid L^{-1}, 1 g propionic

180 acid L^{-1} and 1 g butyric acid L^{-1}. The pH of the PBR was not controlled, yet the influent pH
181 was lowered to 6.5 with 12M HCl to have a final pH of 7.0 in the effluent (pH rises due to
182 VFA consumption). Samples were taken daily to monitor the optical density (660 nm),
183 bacteriochlorophyll peaks (800 nm and 860 nm) to confirm the presence of PNSB, pH,
184 temperature. The remaining sample volume was stored at -20°C for further analysis.

185 **2.3.2 Chemotrophic production in an open aerated bioreactor**

186 A non-axenic aerobic reactor was operated in batch until the stationary phase was reached.
187 The working volume was 2 L and the reactor was covered with aluminum foil to prevent
188 phototrophic growth. Stirring was done with a magnetic stirrer (Carl Roth, Germany) at 700
189 rpm. A pH controller (Consort, Belgium) regulated the pH between 6.9 and 7.1 through the
190 addition of 2 M NaOH and HCl. DO concentration was controlled (Consort, Belgium) by
191 changing the airflow. The k_La was determined through the sulfite oxidation method and was
192 463 ± 66 h^{-1} (Ruchti et al., 1985). The effluent of the PBR was collected as a starter culture
193 for the aerobic reactor. The PBR effluent was first diluted 4.5 times with a fructose-based
194 medium (most promising carbon source according to the batch tests) to a final concentration
195 of 23 g COD L^{-1} (substrate concentration to reach 10 g TSS L^{-1} of biomass; biomass yield
196 0.63 g $COD_{biomass}$ g $COD_{removed}$ Figure 2). The aerobic reactor was then filled with the PBR
197 effluent and the fructose mixture until 2 L. Per batch cultivation, 10 mL of antifoam
198 (Antifoam silicone 414, VWR, USA) was added to the reactor to prevent foam formation
199 (Garrett, 2017).

200 Five sets of experiments were performed. Biological triplicates were obtained for every
201 experiment, based on three sequential production batches using each time fresh PBR effluent.
202 Every batch was operated until the stationary phase was reached by monitoring the optical
203 density at 660 nm. There was 13 ± 3% water evaporation due to aeration and heating of the

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204 reactor (28 °C). Therefore, the reactor volume was adjusted to the initial volume at the end of
205 the experiment. Samples were then taken and stored at -20°C for further analysis.
206 The first experiment was inoculated with aerobic sludge to investigate the productivity
207 and nutritional quality of AHB independently. Two subsequent experiments were inoculated
208 with the effluent of the PBR to explore the effect of DO concentration on productivity,
209 nutritional quality and microbial community structure of the consortium of AHB and PNSB.
210 Two DO concentrations were tested: 0.7 ± 0.1 mg O_2 L^{-1} (experiment 'ii') and 2.0 ± 0.3 mg
211 O_2 L^{-1} (experiment 'iii'). The COD concentration for experiments 'ii' and 'iii' was 16 g COD
212 L^{-1}. Experiment 'iv' was inoculated with the effluent of the PBR and contained a medium
213 with extra trace elements and a higher substrate concentration (23 g COD L^{-1}). The increased
214 COD concentration was not an experimental variable, yet merely used to avoid substrate
215 limitations. Experiment 'v' was inoculated with the effluent of the PBR and an additional 5%
216 aerobic sludge to test if productivities and nutritional quality of the consortium could further
217 be improved.

218 ## 2.4 Analytic procedures

219 The COD was measured using photometric test kits (Merck, Germany) according to the
220 manufacturer's instructions. The biomass yield was determined by dividing produced
221 biomass COD by removed COD. Protein concentration was analyzed by Markwell et al.
222 (1978) (adapted Lowry procedure). TSS and volatile suspended solids (VSS) were measured
223 according to standard methods (Greenberg et al., 1992). Handheld meters were used to
224 determine DO concentration (Hach, USA) and pH (Hanna Instruments, USA). Amino acids
225 were analyzed according to the protocol described by Muys et al. (2019). All EAA profiles
226 were normalized to the diet requirements of shrimp. This was done by dividing the individual
227 EAA values (mg EAA g^{-1} protein) by the shrimp requirements. Values of 1 or higher indicate
228 that the microbial protein source completely covers the shrimp requirements in terms of

229 EAA. Fatty acids methyl esters were prepared by direct esterification according to a modified
230 procedure from Lepage and Roy (1984) and identified with a gas chromatograph (Toi et al.,
231 2013).

232 ## 2.5 Microbial community analyses

233 16S rRNA-gene amplicon sequencing analysis was performed according to De Vrieze et al.
234 (2016) with slight modifications. In brief, DNA extraction was performed by bead beating
235 with a PowerLyzer (Qiagen, Venlo, the Netherlands) followed by a phenol/chloroform
236 extraction. The 16S rRNA gene V3-V4 hypervariable region was then amplified by LGC
237 genomics GmbH (Berlin, Germany). Sequencing was performed using forward primer 341F
238 5'- TCCTACGGGNGGCWGCAG and reverse primer 785R 5'-
239 TGACTACHVGGGTATCTAAKCC(Klindworth et al., 2013). Subsequently, roughly 20 ng
240 amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes.
241 The amplicon pools were purified with one volume AMPure XP beads (Agencourt) to
242 remove primer dimer and other mispriming products, followed by an additional purification
243 on MinElute columns (Qiagen). Lastly, about 100 ng of each purified amplicon pool DNA
244 was used to construct Illumina libraries by means of adaptor ligation using the Ovation Rapid
245 DR Multiplex System 1-96 (NuGEN) (Weithmann et al., 2016). Illumina libraries were then
246 pooled, and size selected by preparative gel electrophoresis. Sequencing was performed on an
247 Illumina MiSeq using v3 Chemistry (Illumina). Read assembly and cleanup were based on
248 the MiSeq SOP described by the Schloss lab (Kozich et al., 2013; Schloss et al., 2011). In
249 brief, mothur (v.1.40.5) was used to assemble reads into contigs, remove chimeras, perform
250 alignment-based quality filtering (alignment to the mothur-reconstructed SILVA SEED
251 alignment, v. 123), assign taxonomy using a naïve Bayesian classifier (Wang et al., 2007) and
252 SILVA NR v132 and cluster contigs into OTUs at 97% sequence similarity. All Eukaryota,
253 Archaea, Chloroplasts and Mitochondria sequences were removed. Moreover, sequences

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254 were also removed if they could not be classified at all (even at (super)Kingdom level). For
255 each OTU representative sequences were picked as the most abundant sequence within that
256 OTU.

257 2.6 Statistical analyses

258 Statistics were performed in R (version 3.4.1) using RStudio (RStudio®, USA) for Windows
259 (R Core Team, 2017). The parametric analysis of variance test and post-hoc pairwise
260 comparisons using the Tukey's range test were performed for multiple comparisons.
261 Normality of data residuals was tested using the Shapiro-Wilk normality test and
262 homogeneity of variances using a Levene's test. If normality was rejected, the non-parametric
263 Kruskal-Wallis rank sum test and post-hoc pairwise comparisons using the Mann-Whitney U
264 test (*p-values* were adjusted using the Bonferroni correction) were performed. The Welch's t-
265 test was conducted in case of heteroscedasticity. A significance level of $p < 0.05$ was chosen.

266 3 Results and discussion

267 3.1 Chemotrophic growth kinetics and yield in batch incubations

268 Batch experiments in 96-Well plates (150 µL) and Erlenmeyer flasks (200 mL) were
269 performed to determine the chemotrophic growth kinetics of PNSB. It was the objective to
270 assess the effect of carbon source and PNSB species on the growth rate, metabolic flexibility
271 to switch from photo- to chemotrophic conditions, biomass yield and protein content.

272 Growth rates of the preliminary 96-Well plate screening showed that PNSB preferred
273 carbohydrates (growth rates $p < 0.05$) over VFA and alcohols during chemotrophic
274 cultivation in contrast to their phototrophic metabolism where they favor VFA (Blankenship
275 et al., 1995). More specifically, fructose resulted in significantly higher growth rates ($p <$
276 0.05) compared to the other carbon sources. Only *Rb. sphaeroides* showed similar growth
277 rates for fructose, VFA and sucrose. No similar studies could be found that compared

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278 multiple PNSB species on their chemotrophic carbon preference. Imam et al. (2013) have
279 studied 190 carbon sources to map out the metabolic and energetic network for *Rb.*
280 *sphaeroides* under both photo- and chemotrophic conditions. During the experiments, only
281 the presence or absence of growth was observed. Consequently, the authors did not derive
282 growth kinetics.

283 This preliminary 96-Well plate screening allowed to select four carbon sources per
284 species as input for the proceeding Erlenmeyer flask tests, which are presented in Figure 1.
285 The results reconfirmed the findings of the 96-Well plate experiment, showing that fructose is
286 an interesting carbon source for the chemotrophic cultivation of the four selected PNSB
287 species. Tests with fructose showed the highest growth rates ($p < 0.05$; excluding *Rps.*
288 *palustris*), lowest lag phase (excluding *Rsp.* rubrum), highest protein content (Figure 2; $p <$
289 0.05; excluding *Rb. sphaeroides*) and highest biomass yield (excluding *Rsp. rubrum).* Ghosh
290 et al. (1994) proposed the use of a combined fructose succinate medium to enhance the
291 pigment formation. However, fructose was not used as a tool to improve growth kinetics. In
292 terms of yield, only Schultz and Weaver (1982) have performed a similar study for fructose,
293 succinate and acetate using *Rsp. rubrum* and *Rb. capsulatus.* Higher biomass yields were
294 observed for fructose (0.72-0.76 g $COD_{biomass}$ g^{-1} $COD_{catabolized}$) compared to other carbons
295 sources such as succinate and acetate (0.50-0.62 g $COD_{biomass}$ g^{-1} $COD_{catabolized}$) for both
296 species in line with our results.

297 A lag phase ranging from 4 to 49 hours was overall observed with *Rb. sphaeroides* and
298 *Rsp. rubrum* the least metabolically flexible and *Rb. capsulatus* the most metabolically
299 flexible to adapt from photo- to chemotrophic conditions. The 3-species synthetic community
300 had a lower lag-phase compared to the individual species (Figure 1). Therefore, a facultative
301 mutualistic association could have occurred within the community (Little et al., 2008). This is
302 in contrast with photoheterotrophic PNSB growth, where competitive or antagonistic

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303 interactions were observed with negative effects on the overall growth rate (Alloul et al.,
304 2019). The lag phase of the AHB culture was significantly lower than for the PNSB because
305 these microorganisms did not need to switch between metabolisms. Ghosh et al. (1994) also
306 comments that a lag phase does occur during the switch from photo- to chemotrophic
307 conditions. It also might have been possible that the lag phase in our study was enhanced by
308 the continuous phototrophic cultivation of PNSB. Sabaty et al. (1993) found that respiratory
309 activity is inhibited by continuous illumination of *Rb. sphaeroides.* Similar effects are
310 feasible for *Rb. capsulatus, Rps. palustris* and *Rsp. rubrum*. A notable observation was the
311 formation of pigments during the dark for all PNSB species on all carbon sources. This was
312 already discovered for *Rsp. rubrum* and is triggered by low aeration levels (Ghosh et al.,
313 1994).

314 *Rb. capsulatus* along with fructose as carbon source was chosen for the 'hybrid' reactor
315 experiments due to the lowest lag phase and additionally its high biomass yield and protein
316 content.

317 3.2 Two-stage photo- and chemotrophic reactor cultivation

318 First, the results of the PBR operated semi-continuously with *Rb. capsulatus* as inoculum and
319 a VFA based medium are described. Secondly, the results of the aerobic reactor, operated in
320 batch mode using the PBR effluent as inoculum and a medium with fructose as a carbon
321 source is discussed.

322 3.2.1 Stable phototrophic cultivation of PNSB

323 A non-axenic semi-continuous PBR, used as a starter culture for the aerobic reactor, was
324 operated as chemostat at an SRT of 0.93 ± 0.1 d for 87 days. Overall, TSS concentration and
325 protein productivity and protein content were steady overtime at respectively 1.16 ± 0.23 g
326 TSS L^{-1}, 0.64 ± 0.11 g protein L^{-1} d^{-1} and 54 ± 2 g protein 100 g^{-1} TSS. Literature values for

327 the protein content of *Rhodobacter* species are between 30-50 g protein 100 g^{-1} TSS, which is
328 comparable to the PBR results in this study (Capson-Tojo et al., 2020).

329 Results of microbial community analysis (Figure 3) showed a high PNSB abundance
330 (93-97%), and low diversity (Shannon index: 0.2-0.4; diversity index: 1.2-1.5). This indicates
331 that the PBR allowed selective and stable production of PNSB overtime under phototrophic
332 conditions, in agreement with previous literature (Hülsen et al., 2016a; Hülsen et al., 2016b).
333 The main competitor genera were *Dysgonomonas* spp. and *Acinetobacter* spp., both gram-
334 negative bacteria with an abundance of respectively between 0.8-3.5% and 0.4-1.7%. This is
335 in agreement with our earlier work showing that *Acinetobacter* spp. are competitors for
336 phototrophically cultivated PNSB (Alloul et al., 2019).

337 Overall, the PBR showed a stable PNSB production over time with a steady biomass
338 concentration, protein productivity, biomass yield and PNSB community (Figure 3). The
339 results confirm that the advantages of phototrophic cultivation are selectivity and high
340 biomass yield (0.97 ± 0.15 g $COD_{biomass}$ g^{-1} $COD_{removed}$).

341 **3.2.2 Chemotrophically maximizing protein productivity**

342 Productivity and biomass yield of the aerobic reactor are presented in Figure 4. The
343 nutritional quality was evaluated based on the EAA (Figure 5) and fatty acid profile (Figure
344 6). EAA were compared to fishmeal and shrimp requirements. Fatty acids were compared to
345 fish oil. These choices were made because the authors anticipate that microbial protein will
346 first be a substitute to aquaculture ingredients such as fishmeal (€ 2 kg^{-1} protein) due to its
347 higher price compared to ingredients for farm animals such as soybean meal, which has a
348 market price of 0.7 kg^{-1} protein (IndexMundi, 2019).

349 The results in Figure 4 compare the individual production of PNSB (PBR) and AHB
350 (aerobic reactor) with the 'hybrid' system (i.e. aerobic reactor inoculated effluent PBR).
351 Protein productivity was up to 10 'times higher for the 'hybrid' system (experiment ii-iv)

352 compared to the PBR, yet biomass yield (0.53 ± 0.02 g $COD_{biomass}$ g^{-1} $COD_{removed}$) was half of
353 that of the PBR (0.97 ± 0.03 g $COD_{biomass}$ g^{-1} $COD_{removed}$) due to aerobic oxidation of fructose
354 to CO_2. For axenic PNSB cultures, only Zeiger and Grammel (2010) have studied
355 chemotrophic growth of *Rsp. rubrum* and reached a productivity of 13 g TSS L^{-1} d^{-1}, slightly
356 higher than our two-stage photo- and chemotrophic system (12 g TSS L^{-1} d^{-1}).

357 The individual AHB production process (experiment ‘i’) had a protein productivity
358 which was 1.4 times higher (7.4 ± 0.4 g protein L^{-1} d^{-1}) compared to the experiment with the
359 ‘hybrid’ system inoculated with PNSB (5.4 ± 0.6 g protein L^{-1} d^{-1}; ‘iv’). AHB have a shorter
360 lag phase than PNSB as they do not need to switch between a photo- and chemotrophic
361 metabolism (Figure 1). However, the ‘hybrid’ system with the PNSB starter culture
362 (experiment ‘ii-iv’) had a better nutritional quality compared to the AHB starter culture. The
363 protein content of the experiment with the PNSB inoculum was 46-71 g protein 100 g^{-1} TSS
364 vs. 36 ± 5 g protein 100 g^{-1} TSS for the AHB inoculum. The ‘hybrid’ system with the PNSB
365 starter culture had also no limitations in EAA for shrimp (Figure 5). On the contrary, the
366 AHB inoculum observed methionine and cysteine, and also phenylalanine and tyrosine
367 limitations relative to shrimp requirements.

368 Another nutritional parameter where the ‘hybrid’ system (experiment ‘ii-iv’)
369 outperformed the individual AHB process (experiment ‘v’) was the fatty acids composition
370 (Figure 6). Experiment ‘ii-iv’ with the PNSB inoculum contained 6-7 g fatty acids 100 g^{-1}
371 TSS compared to 2 g fatty acids 100 g^{-1} TSS for the AHB inoculum. Remarkably, the PBR
372 biomass or the aerobic reactor with the PNSB starter culture were also rich in vaccenic acid
373 (18:1(n-7)), a fatty acid already known to be abundantly present in PNSB biomass
374 (Blankenship et al., 1995; Imhoff, 1991). However, previous literature designated 18:1 as
375 specific for PNSB, yet our results showed that it is the fatty acid 18:1(n-7).

376 Experiment 'iii', at high DO concentration (2.0 ± 0.3 mg O_2 L^{-1}), showed a slightly
377 higher protein productivity than the reactor operated at low DO levels (0.7 ± 0.1 mg O_2 L^{-1}).
378 However, protein content was for both conditions 71 g protein 100 g^{-1} TSS ($p > 0.05$). In
379 terms of EAA composition, values were compared to shrimp requirements. The low DO
380 concentration showed methionine and cysteine limitations, which were not observed for the
381 high DO concentration. This may have been linked to the higher abundance of *Rb. capsulatus*
382 for the low DO concentration, which also showed methionine and cysteine limitations. The
383 fatty acid profiles were comparable, and both conditions contained negligible amounts of
384 essential fatty acids (Figure 6).

385 Results of the microbial community analysis showed that the 'hybrid' system enabled
386 to produce a consortium containing a relative PNSB abundance up to 10% and around 90%
387 for AHB (Figure 3). The highest PNSB abundance was observed for experiment 'ii', which
388 was operated at the low DO concentration of 0.7 mg O_2 L^{-1}. Productivity results show an
389 increase from 2.6 to 5.4 g protein L^{-1} d^{-1} for experiments 'ii' to 'iv' (Figure 4). At the same
390 time, PNSB abundance decreased from 10% to 3%, still in line with the objectives of this
391 research (high proportion of AHB and a low proportion of PNSB). Nonetheless, a higher
392 abundance of PNSB is more favorable. This might be possible by acclimatizing them to
393 oxygen, thereby, further enhancing the value of the product. PNSB have difficulties to rapidly
394 initiate growth due to the inhibition of the respiratory activity by continuous illumination of
395 the PBR as observed by Sabaty et al. (1993) for *Rb. sphaeroides*. Another type of PBR open
396 to air such as a raceway reactor conventionally used for microalgae cultivation (Alloul et al.,
397 2020), could in principle enable PNSB to adapt to oxygen and might prevent the inhibition of
398 the respiratory activity in the subsequent chemotrophic production step. Future research
399 should explore this.

400 This study shows that AHB and PNSB can be produced through a two-stage photo- and
401 chemotrophic production system. However, cultivating AHB and PNSB separately followed
402 by product blending might also be an option. A preliminary cost estimation based on input
403 parameters from other work (Alloul et al., 2020; Alloul et al., 2019), showed that separately
404 cultivating AHB (aerobic reactor) and PNSB (PBR) amounts to a production cost of
405 respectively € 5 kg^{-1} protein and € 27 kg^{-1} protein. This would thus result in a total production
406 cost of € 7 kg^{-1} protein considering a product of 90% AHB and 10% PNSB. On the contrary,
407 the 'hybrid' system would result in a production cost of € 5 kg^{-1} protein or 30% lower than
408 when the individual microbial products are blended (90% AHB and 10% PNSB). The savings
409 for the 'hybrid' system are due to a lower PBR volume compared to an individual PNSB
410 production process. In the two-stage process, the PBR is only used to cultivate the PNSB
411 starter culture and the actual production occurs in the aerobic reactor. A tubular PBR
412 contributes to 50% of total costs. Therefore, decreasing the PBR volume can significantly
413 influence the final production costs. A thorough production cost assessment is, nonetheless,
414 needed to further validate the benefits of the two-stage system.

415 ## 4 Conclusions

416 *Rb. capsulatus* grown on fructose had the best growth performance and was, therefore, the
417 best starter culture/carbon match for the two-stage photo- and chemotrophic systems. The
418 biomass from the two-stage systems had an improved protein- and fatty acid content and
419 amino acid profile (46-71 g protein 100 g^{-1} TSS; no EAA limitations; 9 g fatty acids 100 g^{-1}
420 TSS) vs. one-stage AHB production (36 g protein 100 g^{-1} TSS; EAA limitations; 3 g fatty
421 acids 100 g^{-1} TSS). The consortium contained up to 10% PNSB and production costs were
422 30% lower vs. individual AHB and PNSB cultivation followed by blending.
423 E-supplementary data of this work can be found in online version of the paper.

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Figure captions

Figure 1 Aerobic batch test of purple non-sulfur bacteria (PNSB) cultures and aerobic heterotrophic bacteria (AHB) showing maximum specific growth rate (left y-axis) and lag phase (right y-axis). PNSB were phototrophically pre-cultivated on a volatile fatty acid mixture. Tests were performed in Erlenmeyer flasks. Carbon sources were selected based on a 96-Well plate screening. Error bars show standard error (n=3).

Figure 2 Aerobic batch test of four purple non-sulfur bacteria showing protein content (left y-axis) as share of total suspended solids (TSS) and biomass yield expressed in chemical oxygen demand (COD; y-axis). PNSB were phototrophically pre-cultivated on a volatile fatty acid mixture. Tests were performed in Erlenmeyer flasks. Error bars show standard error (n = 3).

Figure 3 Microbial community composition, purple non-sulfur bacteria (PNSB) abundance and diversity parameters such as Shannon index and diversity index which is the exponential of the Shannon index. The photobioreactor (PBR) was inoculated with *Rhodobacter capsulatus* (in orange) and the aerobic reactor was inoculated with the effluent of the PBR and/or aerobic sludge as aerobic heterotrophic bacteria (AHB) inoculum.

Figure 4 Productivity of photobioreactor and aerobic reactor runs (left y-axis) as protein, non-protein volatile suspended solids (VSS) and fixed suspended solids (FSS) along with biomass yield (right y-axis). *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time and effluent of photobioreactor as starter culture of aerobic reactor. All aerobic reactor experiments were performed in biological triplicates. AHB: aerobic heterotrophic bacteria. Error bars show standard error (n= 3).

Figure 5 Essential amino acid (EAA) content in microbial biomass (g EAA 100 g^{-1} $protein_{biomass}$) relatively to juvenile shrimp requirements (g EAA 100 g^{-1} $protein_{feed}$) for the photobioreactor (Penaflorida, 1989), aerobic reactor with PBR effluent as starter culture and aerobic reactor with aerobic heterotrophic bacteria (AHB) as starter culture originating from aerobic brewery sludge. Values of 1 or higher indicate that the microbial protein source completely covers the shrimp requirements in terms of EAA. *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time. *Rhodobacter capsulatus* as inoculum of photobioreactor operated at 0.93 d sludge retention time.

Figure 6 (A) Fatty acid profile (left y-axis) and total fatty acid content along with of 18:1 (n-7) or 11-Octadecenoic known as a marker fatty acid for PNSB (right y-axis). A pure *Rhodobacter capsulatus* species was used to analyze fatty acids. Fish oil composition based on Trushenski et al. (2011). AHB: aerobic heterotrophic bacteria.

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Highlights

(i) Aerobic grown purple non-sulfur bacteria (PNSB) prefer fructose as carbon source

(ii) *Rhodobacter capsulatus* grown on fructose had the best growth performance

(iii) The consortium contained 10% PNSB and 90% aerobic heterotrophic bacteria (AHB)

(iv) Cocultivating AHB & PNSB improved the amino acid profile vs. separate cultivation

(v) Cocultivating AHB & PNSB resulted in 30% lower costs vs. separate cultivation

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Credit Author Statement

Abbas Alloul: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration
Maarten Muys: Formal analysis; Writing - Review & Editing
Nick Hertoghs: Investigation, Formal analysis
Frederiek-Maarten Kerckhof: Formal analysis, Writing - Review & Editing
Siegfried E. Vlaeminck: Conceptualization, Supervision, Writing - Original Draft, Writing - Review & Editing

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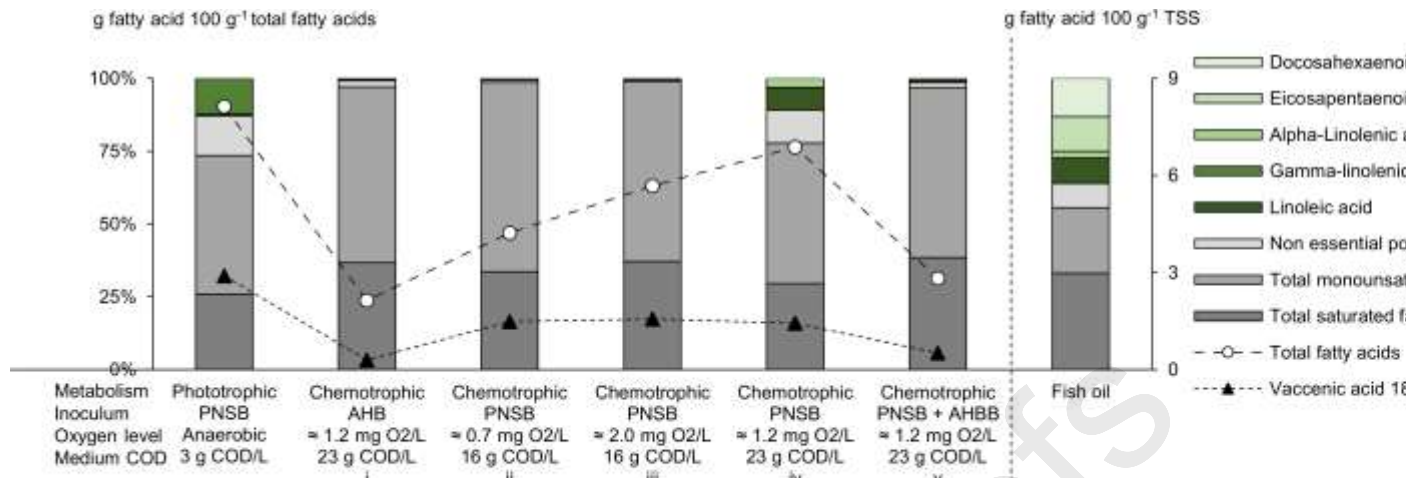
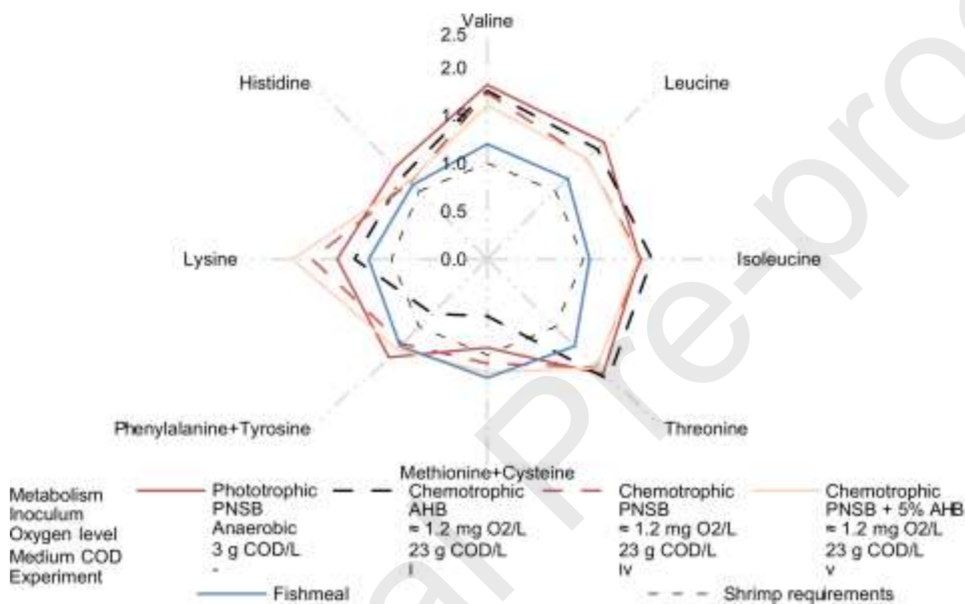
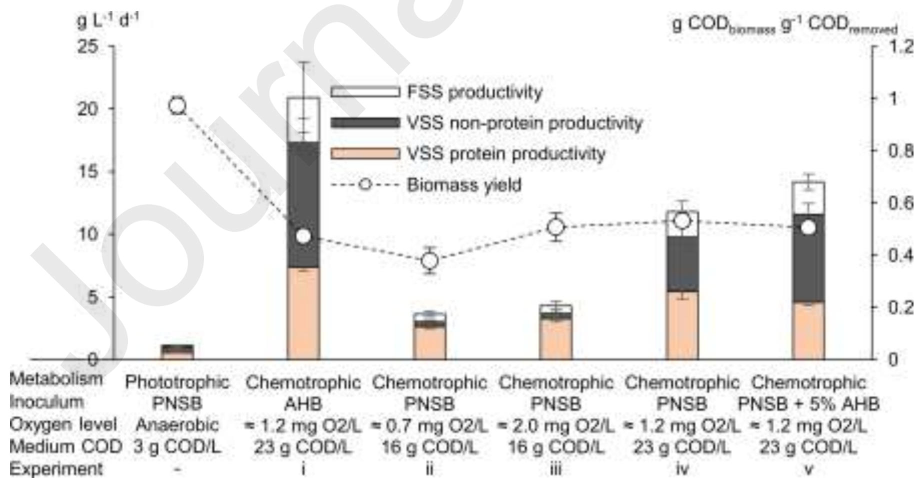
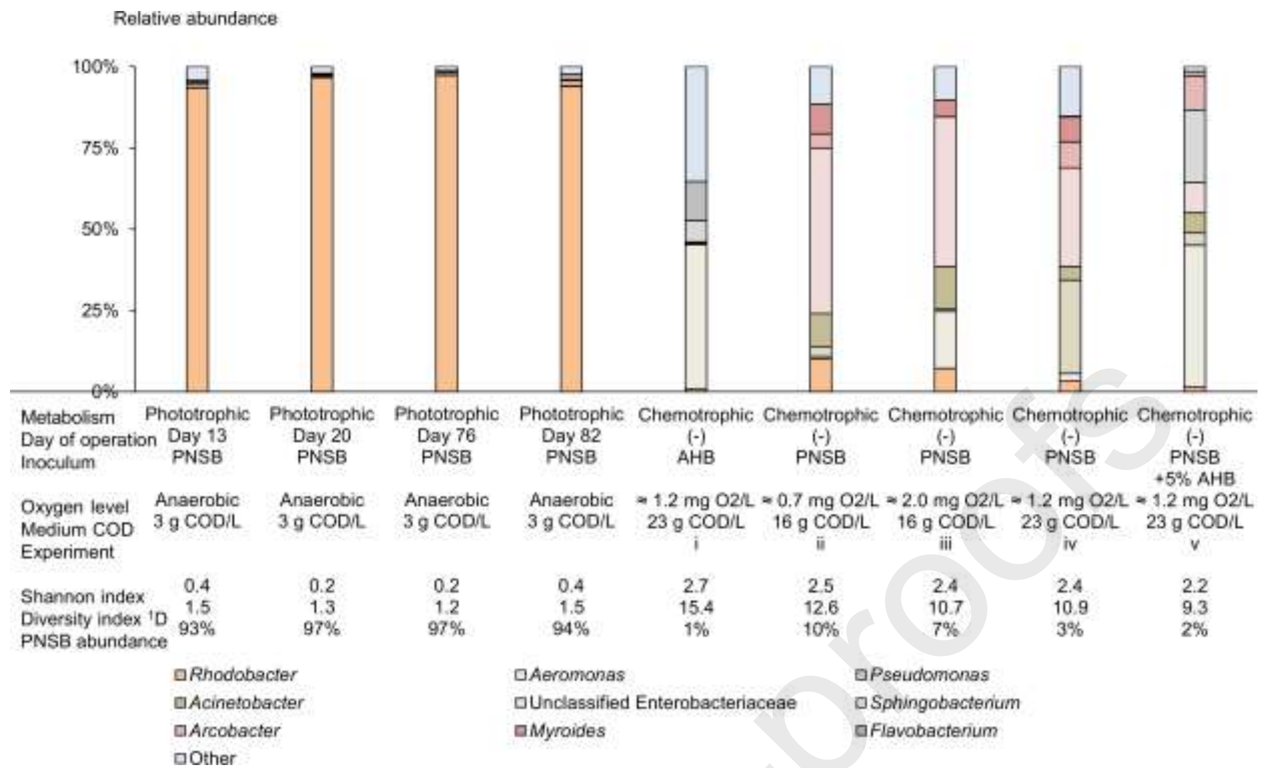
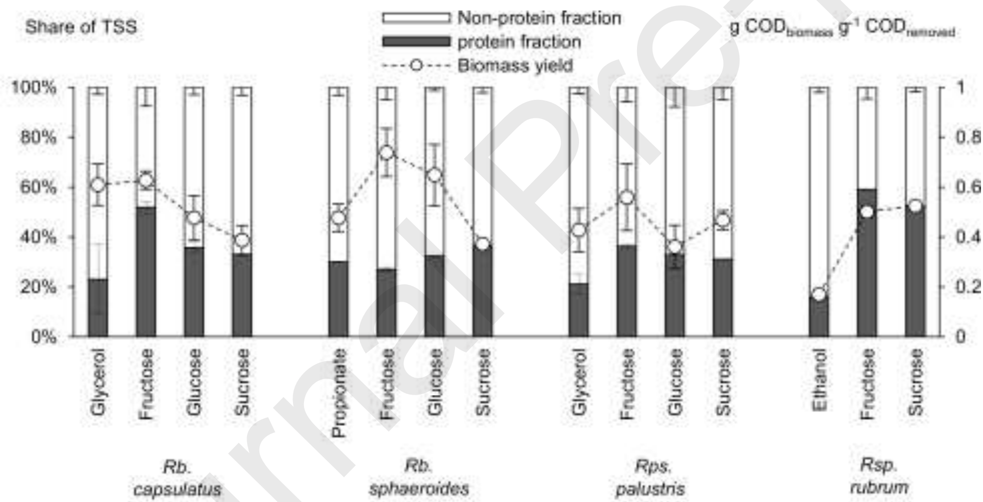

g fatty acid 100 g-1 total fatty acids
g fatty acid 100 g-1 TSS
100%
75%
50%
25%
0%
Metabolism
Inoculum
Oxygen level
Medium COD
Phototrophic PNSB Anaerobic 3 g COD/L
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 0.7 mg O2/L 16 g COD/L ii
Chemotrophic PNSB ≈ 2.0 mg O2/L 16 g COD/L iii
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + AHBB ≈ 1.2 mg O2/L 23 g COD/L v
Fish oil
Linoleic acid
Total fatty acids


Valine
Leucine
Isoleucine
Threonine
Methionine+Cysteine
Phenylalanine+Tyrosine
Lysine
Histidine
2.5
2.0
1.0
0.5
0.0
Metabolism
Inoculum
Oxygen level
Medium COD
Experiment
Phototrophic PNSB Anaerobic 3 g COD/L -
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + 5% AHB ≈ 1.2 mg O2/L 23 g COD/L v
Fishmeal
Shrimp requirements


g L-1 d-1
g CODbiomass g-1 CODremoved
FSS productivity
VSS non-protein productivity
VSS protein productivity
Biomass yield
Metabolism
Inoculum
Oxygen level
Medium COD
Experiment
Phototrophic PNSB Anaerobic 3 g COD/L -
Chemotrophic AHB ≈ 1.2 mg O2/L 23 g COD/L i
Chemotrophic PNSB ≈ 0.7 mg O2/L 16 g COD/L ii
Chemotrophic PNSB ≈ 2.0 mg O2/L 16 g COD/L iii
Chemotrophic PNSB ≈ 1.2 mg O2/L 23 g COD/L iv
Chemotrophic PNSB + 5% AHB ≈ 1.2 mg O2/L 23 g COD/L v





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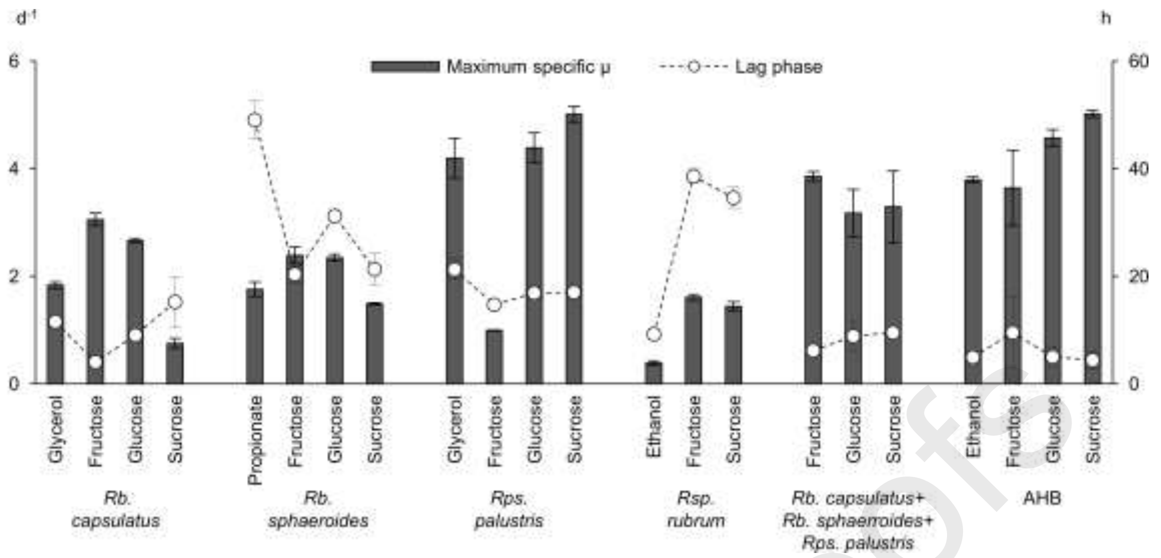

Journal Pre-proofs