Effects of light / dark diel cycles on the photoorganoheterotrophic metabolism of *Rhodopseudomonas palustris* for differential electron allocation to PHAs and H₂

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Abstract

Light/dark cycles can impact the electron distribution in *Rhodopseudomonas palustris*, a hyperversatile photoorganoheterotrophic purple non-sulfur bacterium (PNSB). Dynamic conditions during diel cycles are important for the physiology of PNSB, but the coupling between illumination patterns and redox balancing has not been extensively studied. For survival and growth, *Rhodopseudomonas* has developed different mechanisms to allocate electrons under dynamic growth conditions. Products such as hydrogen and poly- β -hydroxyalkanoates (PHAs) can form alternative electron sinks. A continuous culture, fed with a balanced nutrients medium, was exposed to three different conditions: 24 h continuous infrared illumination, 16h light/8h dark, and 8h light/16h dark. Light and dark phase durations in a cycle determined the energy availability level (light) and the attainment of a stationary state. Under long dark phases, the acetate substrate accumulated to levels that could not be depleted by growth in the light. Under short dark phases, acetate was rapidly consumed in the light with most of the phototrophic growth occurring under acetate-limiting conditions. Under diel cycles, substrate uptake and growth were unbalanced and *Rhodopseudomonas* shunted the excess of carbon and electron flow first toward PHAs production. Only secondarily, when PHA storage got saturated, the electron excess was redirected toward H₂. A numerical model described well the dynamics of biomass and nutrients during the different light/dark cycle regimes. The model simulations allowed determination of stoichiometric and kinetic parameters for conversion by *Rhodopseudomonas*. Understanding the inherent process dynamics of diel light cycles in purple sulfur bacteria cultures would enable optimization procedures for targeted bioproduct formation.

Importance

Purple non-sulfur bacteria (PNSB) are important anoxygenic phototrophic microorganisms that take part in numerous environmental processes, based on their metabolic versatility. *Rhodopseudomonas*

palustris is a model photosynthetic bacterium of the PNSB guild. Light cycles influence deeply its physiology. Poly- β -hydroxyalkanoates (PHAs) and biohydrogen are two of the most studied metabolic products of *Rhodospseudomonas*, because of their biotechnology potential besides involvement in carbon and electron allocations in its metabolism. Their production mechanisms have often been described as competitive, but the rationale behind the production of one or the other compound has not been elucidated. Here, we found that under light / dark cycles an excess of organic substrate was first directed toward PHAs production, and only when this pathway was saturated H₂ was produced. Understanding the dynamics of carbon and electron allocation under intermittent light cycles enhances our knowledge on PNSB metabolisms and paves ways to manage the formation of targeted bioproducts.

Keywords

purple non-sulfur bacteria; photoorganoheterotrophy; electron distribution; biohydrogen; polyhydroxyalkanoates; diel cycles; mathematical modeling

Introduction

Purple non-sulfur phototrophic bacteria (PNSB) form a guild of hyper-versatile anoxygenic phototrophs (1), able to grow on different organic and inorganic substrates (2). *Rhodopseudomonas palustris* is one model PNSB (3). It can produce compounds of industrial interest, such as single cell proteins (4), carotenoids (5), hydrogen gas (H₂; used as biofuel) and poly-β-hydroxyalkanoates (PHAs; used as bioplastics) (6,7). Under light and in presence of organic carbon, *Rhodopseudomonas* grows photoorganoheterotrophically even in absence of external electron acceptors. Under dark, growth is possible on sugars with external electron acceptors (as nitrate, trimethylamine-N-oxide or dimethyl sulfoxide) (8,9).

Light is crucial for phototrophy by providing energy to cells. PNSB capture the photonic energy and couple the light-driven oxidation of the photopigments to an electron transfer through membrane bound-enzymes. The trans-membrane gradient of hydrogen protons (H⁺) supports ATP synthesis through cyclic photophosphorylation (10). NADH is mainly produced during catabolic processes. In some cases, a reverse electron flow takes places, and the NADH-dehydrogenase catalyze the proton transfer from the ubiquinone pool to NAD⁺. The control of redox balance is crucial for cell survival and growth. The ratio between NADH/NAD⁺ is important for intracellular redox homeostasis (11). PNSB generate NADH in three different ways (Figure 1), namely: 1) anabolic processes (12), 2) light driven reactions through the quinone pool (1); 3) reverse electron transfer (13).

To cope with possible redox imbalances, mainly arising in anabolic processes, PNSB redistribute the electrons toward different routes (14). In *Rhodopseudomonas*, the first and most important electron sink is the biomass itself, that receives the intermediates of the tricarboxylic acid cycle and the NADPH produced there (15). Secondly, the Calvin-Benson-Bassham (CBB) cycle is constitutively active also under photoorganoheterotrophy as central recycling mechanism of redox

86 cofactors coming from the anabolic process (15,16). Under photoorganoheterotrophic anaerobic
 87 conditions, a surplus of electrons can be disposed through PHA or H₂ production. Both electron
 88 sinks are normally produced under nitrogen limitation. Nitrogenase-mediated H₂ production has
 89 been extensively described (1). H₂ is a side-product of the nitrogen fixation process, that can only
 90 happen when no preferred nitrogen source like ammonium or glutamate is available (17–19).
 91 Hydrogenase-mediated H₂ production has been reported in presence of an oxidized organic
 92 compound (*i.e.* malate) (21). Several bacterial species accumulate PHAs in the cells as a mean of
 93 carbon and electrons storage under normal conditions (22). In lab-scale experiments, to achieve an
 94 over production, these storage polymers are synthesized when acetyl-CoA and NADPH are in
 95 excess but nutrients like nitrogen, sulfur or phosphorus are limiting (23). Similar to other bacteria,
 96 PNSB can produce PHAs (24) as a mean of carbon and electron balance, utilizing the reducing
 97 power to build the storage polymers (25,26). PHAs production in PNSB occurs under balanced
 98 growth, and it increases under nutrient limitations (27). The two processes of H₂ and PHA
 99 production are considered competitive electron dissipation pathways (28,29). However, the hyper-
 100 versatility of PNSB metabolisms has not enabled to identify an univocal response to redox
 101 imbalances.

102

103 In natural environments, phototrophs are subjected to diel (*i.e.*, 24-h period) light / dark cycles.
 104 Since light is responsible for the production of energy and reducing power, the irradiation patterns
 105 impacts the cellular physiology. The light and dark cycles impact cells redox and ATP balance,
 106 subjecting cells to metabolic switches. How it affects the internal electron allocation patterns and
 107 how PNSB respond to these switches remains puzzling. Allocation of reducing power toward the
 108 aforementioned routes is known, but mechanisms that govern the preferential electron flow have not
 109 been explained.

110

111 Here, we aimed to elucidate the effects of diel light / dark cycles on the physiology and electron
112 allocation in *Rhodopseudomonas palustris*, isolated from an in-house PNSB enrichment culture for
113 nutrient removal from wastewater (30). The dynamic conditions were hypothesized to influence the
114 physiology of purple bacteria, as the energy source is intermittently provided. The process mimics a
115 potential natural scenario, where day and night cycles are applied and create a redox imbalance. We
116 elucidated the preferential carbon and electron redistribution toward the most important competitive
117 pathways of PHAs and H₂ formation during diel light / dark cycles.
118

120 **Figure 1.** Schematic representation of the reducing power allocation in *Rhodopseudomonas*. Red lines: electron flow;
121 Black dashed lines: proton transfer; Blue dotted lines: NADH is normally produced in the catabolic processes, whereas
122 NADPH is used in the anabolic processes, such as PHAs formation. Biomass and CBB are the primary electron sinks in
123 PNSB. PHAs and H₂ are two of the other possible electron sinks.

Results

Three regimes for continuous cultivation of *Rhodopseudomonas palustris* were tested: continuous illumination, cyclic 16 h light / 8 h dark, and cyclic 8 h light / 16 h dark. The biomass and CO₂ yields (Y_{XS} and Y_{CX}) and the maximum biomass-specific growth rate (μ_{max}) were estimated using data from the 16 h light / 8 h dark experiment (Table 1). The calibrated model was used to predict the behavior of the 8 h light / 16 h dark cycles.

Biomass production of *Rhodopseudomonas palustris*

Under continuous illumination, the biomass reached a constant concentration of 12.10 ± 0.44 C-mmol L⁻¹, corresponding to 88% of carbon or 99% of reduction equivalents (COD) provided in the influent. Around 11% of the carbon present in the inflow was recovered as CO₂ (0.112 C-mmol L⁻¹) in the off-gas. HPLC results showed no residual acetate in the bulk liquid at steady state.

Under cyclic operations (Figure 2), the system reached a stable behavior with similar changes in concentrations during each cycle. Within a cycle a clear dynamic pattern was observed. Under dark, the biomass did not grow and was only washed-out. The biomass wash-out rate ($F/V \cdot C_X$) was very well represented by the numerical model (Figure 2A,B). The corresponding change in acetate, ammonium and phosphate concentrations during the dark phases matched with the expected change based on the feeding rate. No conversion occurred in the dark.

Under 16-h light, the biomass accumulation exceeded the dilution rate $D=F/V$. An initial growth rate (assumed to be μ_{max}) of 0.15 h⁻¹ was observed, and reached a steady value ($\mu = D = 0.04$ h⁻¹) after 2 h exposed to light (Figure 2A). The acetate concentration reached a minimal steady value when the biomass growth rate achieved the steady state.

Under shorter 8-h light periods, the biomass growth rate did not reach a stationary-state in a cycle, being around 0.1 h^{-1} . Acetate was not depleted at the end of the light phase (Figure 2B). The biomass increase changed from 11 C-mmol L^{-1} on first cycle to 5 C-mmol L^{-1} on fourth cycle. The growth rate μ during the 8 h light period was approximately 0.12 h^{-1} thus lower than the $\mu_{\max}$. The estimated biomass yield on acetate in the light was $0.88 \text{ C-mol X C-mol}^{-1}$ acetate for both the 16 h light and the 8 h light phases, equal to the yield in continuous light regime.

Table 1. Model parameters. The maximum biomass-specific growth rate and yields were fitted to experimental data, while the other parameters were measured during experiments, calculated or obtained from literature.

Parameter name	Symbol	Value	Units	Source
Max. biomass-specific growth rate	$\mu_{\max}$	0.15	$\text{C-mol X} \cdot \text{h}^{-1} \cdot \text{C-mol}^{-1} \text{ X}$	Fitted
Biomass yield	Y_{XS}	0.88	$\text{C-mol X} \cdot \text{C-mol}^{-1} \text{ S}$	Fitted
Ammonium yield	Y_{NX}	0.18	$\text{mol} \cdot \text{C-mol}^{-1} \text{ X}$	Fitted
Phosphate yield	Y_{PX}	0.009	$\text{mol} \cdot \text{C-mol}^{-1} \text{ X}$	Fitted
CO₂ yield	Y_{CX}	0.142	$\text{mol} \cdot \text{C-mol}^{-1} \text{ X}$	Fitted
Reactor radius	R	0.055	m	Measured
Liquid volume	V	1.2	L	Measured
Liquid flow rate	F	0.048	$\text{L} \cdot \text{h}^{-1}$	Measured
Dilution rate	D	0.04	h^{-1}	F/V
Gas volume	V_g	0.3	L	Measured
Gas flow rate	F_g	7.14	$\text{L} \cdot \text{h}^{-1}$	Measured
Inflow acetate concentration	$C_{S,in}$	7	mM	Measured
Inflow ammonium concentration	$C_{N,in}$	4.28	mM	Measured
Inflow phosphate concentration	$C_{P,in}$	0.22	mM	Measured
Light intensity at reactor walls	I_0	300	$\text{W} \cdot \text{m}^{-2}$	Measured
Light extinction coefficient per biomass concentration	ε	0.1	$\text{m}^2 \cdot \text{g}^{-1}$	(17)
Half-saturation coefficient acetate	K_S	0.1	mM	Lower than

in (36) (0.3 mM)

Half-saturation coefficient K_N 0.001 mM (37)

ammonium

Half-saturation coefficient K_P 0.003 mM (38)

phosphate

Half-saturation coefficient light K_I 10 $\text{W}\cdot\text{m}^{-2}$ (39)

CO₂ mass transfer coefficient k_{La} 32 h^{-1} Calculated (SI-1)

Henry coefficient CO₂ H_C 0.03 (at 30°C) $\text{mol}\cdot\text{L}^{-1}\cdot\text{atm}^{-1}$ (40)
0.04 (at 20°C)

159

160 **Carbon, nitrogen and phosphorus assimilation**

161 Under continuous illumination, no residual acetate was detected in the bulk liquid. Under 16 h light,
162 acetate was almost fully depleted, down to a concentration around 1 C-mmol L⁻¹, as also very well
163 represented by the numerical model. Under 8 h light, acetate was still present in the bulk liquid in
164 concentrations ranging from 4 to 1.5 C-mmol L⁻¹. In this case, the model overestimates the rate of
165 acetate consumption, *i.e.* predicting acetate depletion at the end of the light phase.

166

167 Nitrogen and phosphorus sources were provided in excess, and never became limiting. Under light,
168 N and P were consumed, resulting in a decrease in their concentrations (Figure 2C,D). Under dark,
169 the fed N and P accumulated since not assimilated in biomass. At the end of the dark phase, $3.6 \pm$
170 $0.6 \text{ N-mmol L}^{-1}$ and $0.14 \pm 0.01 \text{ P-mmol L}^{-1}$ remained the bulk liquid. N and P dynamics showed
171 irregular behaviour under 16 h light (Figure 2C). Therefore, the Y_{NX} and Y_{PX} were fitted on the 8 h
172 light experiment (Figure 2D). The determined yields ($Y_{NX} = 0.18$, $Y_{PX} = 0.009$) were very close to
173 the theoretical yields resulting from considering the biomass elemental composition ($Y_{NX} = 0.18$,
174 $Y_{PX} = 0.014$) measured in (35). With these yields, the N and P dynamics during the 8 h light cycles
175 were well represented by the model: accumulation under dark and consumption under light.

176

177

178 **Figure 2.** Dynamics of biomass, acetate, ammonium and phosphate concentrations during light-dark cycles: (A),(C) 16
 179 h light / 8 h dark; (B),(D) 8 h light / 16 h dark. Gray areas represent the dark periods. (A),(B) biomass (black circles)
 180 and acetate (red open squares) measured concentrations, with lines being the model results. The biomass concentration
 181 achieved a stationary state, while acetate reached very low (limiting) concentrations after 2 h of the 16 h light periods.
 182 The biomass increased without reaching a steady state during the 8 h light periods, while acetate was not fully
 183 consumed. (C),(D) ammonium (blue diamonds) and phosphate (green triangles) measured concentrations, with lines
 184 being the model results. Concentrations of N and P were not limiting for the system in any illumination phase.

185

186 **CO₂ production follows the biomass growth patterns**

187 The CO₂ production rate was constant at 0.075 ± 0.001 mmol h⁻¹ during continuous illumination
 188 (Figure 3A): the chemostat achieved a stationary operation. Under light/dark cycles, CO₂ emission
 189 in the off-gas decreased in the dark to 0.03 mmol h⁻¹ (Figure 3B). Possibly, the registered baseline
 190 value can be due to the detection limit of the MS instrument. Under 16 h light, CO₂ emission
 191 increased rapidly within the first hour, reaching 0.25 mmol h⁻¹, following the biomass growth rate
 192 with acetate uptake. Once the acetate reached the minimal level, the CO₂ production also decreased

to a stable level of 0.07 mmol h^{-1} after the second hour of illumination, reflecting the steady state operation. The numerical model reproduced the observed trends during light phase, but the residual CO_2 production in the dark phase was not accounted for.

Under 8 h light / 16 h dar, the CO_2 production did not achieve a steady state. In the light phase, the CO_2 production increased with 0.05 to 0.08 mmol h^{-1} during the cycles, reaching a peak after 4 h ($0.88 \pm 0.013 \text{ mmol h}^{-1}$), which was described only qualitatively by the model (Figure 3C). The reason for the discrepancy between model and experimental data is the larger computed acetate consumption rate, which leads to a higher CO_2 formation rate in the model results. In both illumination regimes, the CO_2 production dropped to the same stable value of $0.033 \text{ mmol h}^{-1}$ during the dark phase.

H_2 is produced only during the short light periods

A very low H_2 production was recorded during the continuous illumination with rates of around $0.004 \text{ mmol h}^{-1}$ (Figure 3D). Occasionally, unexplained H_2 spikes were recorded. During the 16 h light experiment, dark-phases hydrogen production was negligible and no specific production pattern was measured in the light (Figure 3E). The very low H_2 concentration may relate to an instrumental offset. However, H_2 was produced constantly during the 8 h light periods (Figure 3F), with an increase of $0.02 \pm 0.01 \text{ mmol h}^{-1}$ per cycle and reaching a maximum of 0.05 mmol h^{-1} . The biomass-specific rate of H_2 production under 8 h light was 10 times higher ($0.156 \text{ mmol H}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$) than under all other conditions ($0.014 \text{ mmol H}_2 \text{ h}^{-1} \text{ g}^{-1} \text{ DW}$).

215

216 **Figure 3.** Dynamics of CO₂, H₂ and PHA during light-dark cycles: (A),(D) continuous illumination; (B),(E),(G) 16 h
 217 light / 8 h dark; (C),(F),(H) 8 h light / 16 h dark. Gray areas represent the dark periods. (A),(B),(C) CO₂ production rate
 218 - measured (red circles) and computed (lines). CO₂ production was constant during continuous illumination, but peaks
 219 appeared during light-dark cycles, correlated with the acetate uptake. (D),(E),(F) measured H₂ production (blue circles).
 220 Constant low level H₂ was produced during continuous illumination and 16 h light cycles, but H₂ production strongly
 221 increased in each light period of the 8 h light cycles. (G),(H) PHAs fluorescent counts per gram biomass in the
 222 light/dark experiments. A PHAs peak was measured at the beginning of each of the 16 h light phases, however less
 223 clear pattern could be detected under 8 h light.

224

225 PHA production follows CO₂ formation under 16 h light but not under 8 h light

226 Due to the low biomass concentration, a traditional PHA extraction and GC quantification (41) was
 227 not possible. Fluorimetry was used to detect PHAs in the biomass, giving a relative quantification
 228 (34). PHAs were not detectable under continuous illumination. A fluorescence count peak was
 229 detectable after 2 h from the light switch in the 16-h light phases ($3.8 \pm 0.5 \cdot 10^5$ fluorescent counts g⁻¹

230 ¹ DW) (Figure 3G), concomitantly to the peak of CO₂ production. The PHAs decreased constantly
 231 after the peak, reaching a baseline at 3·10⁴ fluorescent counts g⁻¹ DW. Under 8 h light / 16 h dark
 232 cycles (Figure 3H), PHA production during light was less pronounced. More PHAs were detectable
 233 in the dark compared to the 16 h light regime. These observations indicate that the diel light regimes
 234 induced PHA formation, with the long light regime generating a typical feast-famine behavior (42),
 235 while the short light regime stimulated a higher PHAs content in the cells.
 236

Discussion

Acetate rather than light drives the metabolic responses of *Rhodopseudomonas*

Light energy source is central for the catabolic processes in PNSB. Organic substrates are used as C-source for biomass synthesis and as electron donors. When no external electron acceptor is present, the difference in degree of reduction between substrate and biomass has to be balanced with internal electron reallocation processes. The dynamics of the change in N and P concentrations were congruent with the biomass formation and their measured yields were close to the elemental biomass composition ($CH_{1.8}O_{0.38}N_{0.18}P_{0.014}$) determined in another study (35). Carbon balances closed for the continuous illumination experiment and for the 16 h light / 8 h dark experiment, but not for the 8 h light / 16 h dark cycles, where the C-balances seemed to indicate a net production (Table 2). PHA granules that were always present in the cells under 8 h light / 16 h dark may have led to an absorbance overestimation of the biomass present in the reactor.

Table 2. Distribution of the carbon sources and carbon balances under different illumination conditions

Light/dark regimes	Carbon inputs (C-mmol h ⁻¹)		Carbon outputs (C-mmol h ⁻¹)			Carbon balance (% C)	Electron balance (% COD)
Component	Acetate	Acetate	Biomass	CO ₂	Σ out		
Phase	Influent	Effluent	Effluent	Off-gas			
24 h light	0.66±0.05	<LOD ¹	0.58	0.07±0.001	0.65	98%	98%
16 h light	0.66±0.05	0.11±0.01	0.57±0.01	0.08±0.01	0.75±0.01	115±2%	113±1%
8 h dark		0.10±0.02	0.51±0.03	0.07±0.02	0.68±0.06	103±9%	102±9%
8 h light	0.61±0.006	0.28±0.03	0.62±0.05	0.07±0.01	0.97±0.05	158±8%*	159±9%*
16 h dark		0.19±0.07	0.57±0.12	0.03±0.002	0.79±0.15	130±25%*	137±27%*

¹limit of detection; * the overvaluation of the carbon and electron balance can be due to an overestimation of the biomass concentration. The absorbance measures taken might have influenced by the presence of PHAs inside the cells.

Light / dark cycles occur daily in natural environments. Phototrophic organisms have adapted to

cope with imbalances in energy supply. Purple bacteria have higher biomass production under light / dark cycles (43,44). H₂ production is increased under discontinuous illumination, following the biomass trend (45,46).

258

Here, the light cycles are important not only for the energy that light provides, but most importantly for the time available for the cultures to metabolize the nutrients. The light intensity at the surface of the reactor was 300 W m⁻². Considering the attenuation due to the biomass concentration and the reactor depth, the minimum calculated light available was 180 W m⁻² (Figure SI-4). Under any given condition, the available light intensity was not limiting the microbial metabolism since always exceeding the half-saturation coefficient for light, defined at $K_i = 10 \text{ W m}^{-2}$ (39). Purple bacteria adapt the number and type of photosynthetic unit components based on the different light intensities (47), but Imam et al. (48) reported that light may be saturating for PNSB growth already at 100 W m⁻². We can therefore assume that the irradiance intensity used in our study during the light periods did not deeply affect the metabolic state of the cells.

269

The continuous cultivation system was initially set-up based on the stoichiometric and kinetic parameters derived from batch experiments. In the continuously illuminated chemostat, the biomass reached a steady-state, acetate was limiting (i.e., low concentrations), and the biomass growth rate equaled to the dilution rate (0.04 h⁻¹). Once the light/dark cycles were applied, a disturbance of the steady-state was immediately observed. Under long (16 h) illumination, the biomass consumed the acetate for growth and after an initial increase of the growth rate the steady-state conditions were restored for the remaining two thirds of the light cycles (with a specific growth rate again equal to the dilution rate). Longer (16 h) dark periods led to an increased accumulation of acetate in the bulk liquid (twice as high as in the 8 h dark), since acetate was fed twice longer and there was no consumption in the dark. Due to the shorter light (8 h light) periods, acetate was not fully consumed at the end of the light phases. Therefore, acetate was no more the growth rate limiting compound

281 and the light period was too short to reach a steady state. The growth rate in the light phase
 282 exceeded the value of the imposed dilution rate, leading to a transient accumulation of biomass
 283 (Figure 2B). The modelled biomass dynamics agreed with the measurements, fitting quantitatively
 284 the values for the 16 h light experiment, where the exact steady state values were obtained. When
 285 using the same model parameters for the short (8 h) illumination experiment, the calculated amount
 286 of biomass formed during the light periods corresponded well to the values measured only for the
 287 second and later cycles, while for the first cycle the model underestimated the biomass formed. The
 288 disagreement may be related to the carbon imbalance reported in Table 3. Still, given longer
 289 illumination in the last model cycle would allow the biomass to reach exactly the same steady state
 290 as in the longer light period experiment (Figure SI-3) after about 10 h light and acetate attained the
 291 minimum.

292
 293 The average acetate consumption rates under 16 h light were $0.08 \text{ C-mol h}^{-1} \text{ L}^{-1}$ (Figure SI-5) and
 294 under 8 h light were $0.15 \text{ C-mol h}^{-1} \text{ L}^{-1}$ (Figure SI-6). Initially, the biomass grew at the maximum
 295 substrate uptake rate. If the light phase was too short (as in the 8 h light periods), acetate was not
 296 fully consumed ($2.5 \pm 1.1 \text{ C-mmol L}^{-1}$) and the biomass grew at its maximum growth rate.

298 **PHA synthesis patterns reflect the metabolic state of the cells**

299 PHAs can be formed under dynamic conditions or because of imbalance in the degree of reduction
 300 between biomass and carbon source. PHAs constitute carbon and energy stocks (49,50). In
 301 chemostat conditions, carbon was continuously fed to the cultures and cells did not store PHAs.
 302 PHA synthesis in purple bacteria has primarily been reported under nitrogen limitation, as an
 303 intrinsic mechanism for the redistribution of carbon excess. PHA accumulation is conventionally
 304 reported under growth-limiting conditions (51,52) when the carbon sources are available, but the
 305 nutrients (such as N, P or S) to produce cellular components are limited resulting in acetyl-CoA
 306 accumulation in the cells.

307

308 During the anabolic reactions, *Rhodopseudomonas* incorporates acetate in the form of acetyl-CoA
309 and releases at the same time CoA. In highly active cells, the levels of acetyl-CoA are low, but the
310 levels of CoA are high. In contrast, in non-growing cells, acetyl-CoA is not utilized in anabolic
311 processes and accumulates in the cells (53). A high acetyl-CoA to CoA ratio and the presence of
312 NADPH are required for the initiation of the PHA pathway (54).

313

314 The ratio between carbon sources and other nutrients is important to maintain the balance between
315 substrate uptake rate and growth rate. The inflow was defined based on the biomass composition of
316 purple bacteria (38), with a C:N ratio of 5.68:1 mol/mol was used in the medium. Nitrogen was
317 provided in excess preventing N-limitation imbalance. Consequently, under continuous
318 illumination, no production of PHAs was detected.

319

320 After a period of darkness, the biomass was subjected to an excess of light and nutrients (acetate,
321 ammonium, phosphate) resulting in an increased substrate uptake rate. For the first 2 h after
322 switching on the 16-h light phase, the growth rate was close to the previously measured maximal
323 growth rate $\mu_{\max}$ of 0.15 h⁻¹. After 3 h, when acetate was no more in excess, the growth rate
324 stabilized again at the dilution rate value of 0.04 h⁻¹. When shorter (8 h) light periods were applied,
325 the initial growth rate was ~0.1 h⁻¹. Under both conditions, cells exhibited growth rates close to
326 $\mu_{\max}$. This resulted in the production of reducing power, which, along with the NADH produced in
327 the photosynthetic processes, became in excess and had to be reallocated. As reported by Kanno et
328 al. (55), the photosynthetic units are not disassembled in the dark, even under starvation. These are
329 readily available, once the light conditions are restored, to produce ATP and NADH for the
330 biosynthetic processes. This, linked to the prompt availability of the enzymes for PHAs formation
331 that are constitutively expressed (56), leads to the immediate production of PHAs. Under 16 h light,
332 a peak of fluorescent counts was observed 2 h after the light switch, but it decreased to baseline

levels (steady state) once the growth rate stabilized again at 0.04 h^{-1} . The fluorescent counts decreased and no H_2 production was observed. Cells reached a maximal capacity of substrate uptake, though not coupled to a maximal growth rate, similarly to what is described in (57).

Under 8 h light / 16 h dark cycles, acetate was not completely consumed during illumination and increasingly accumulated during the dark phases, resulting in a further imbalance in the redox state of the cells. The redox imbalance generated by the continuous presence of acetate, rather than the illumination conditions alone, led to a more constant production of PHAs. The cells were highly active, with a growth rate close to the maximal growth rate, leading to a low availability of CoA that resulted in PHAs formation. Only when the PHAs pool got saturated the NADPH pool was further increased, and H_2 production became possible.

H_2 production is a secondary pathway of electron dissipation

The H_2 production rates here reported are low compared to other studies that have exposed PNSB to similar light intensities (48). However, our experimental conditions were not designed to stimulate H_2 production. H_2 can be produced either via the nitrogenase system, either via the ferredoxin-hydrogenase system. Ammonium is known to inhibit N_2 fixation in photosynthetic bacteria. It also effectively prevents photoproduction of H_2 , due to inhibition and inactivation of nitrogenase (18). The presence of ammonium in non-limiting amounts in the medium indicated that potentially H_2 production was driven by the hydrogenase rather than by the nitrogenase. The molar C:N ratio (5.7) was 7 times lower than in most other studies on H_2 production (average C:N ratio 40) (58). Nonetheless, the H_2 production rate per gram biomass during the 8 h light phases was around 11 times higher than in all other conditions ($0.156 \text{ vs } 0.014 \text{ mmol h}^{-1} \text{ g}^{-1} \text{ DW}$), indicating that H_2 production acts as further electron dissipation pathway. A similar H_2 production pattern under alternate irradiation in PNSB cultures has been found (59), with H_2 production only during the light phases. H_2 production was not integrated in the numerical model, since H_2 did not contribute

359 significantly to the stoichiometric balance and kinetics.

360

361 The absence of relevant H₂ production over 16 h light (0.004 ± 0.0004 mmol h⁻¹) and its low but
362 constant production during 8 h light (0.025 ± 0.009 mmol h⁻¹) indicates that the H₂ sink did not play
363 a major role in the electron redistribution patterns.

364

365 Overall, PNSB are one of the most versatile guilds of microorganisms. They present consistent
366 differences in their metabolisms, interspecies and intraspecies. We presented a comprehensive
367 explanation of the underlying mechanisms of electron allocation using quantitative biotechnology
368 and metabolic modelling. The numerical model represented well the biomass and nutrient dynamics
369 during the light/dark cycles of different durations. It allowed for determining stoichiometric and
370 kinetic parameters of the *Rhodospseudomonas*. It can be concluded that:

- 371 1) Under light-saturating conditions and continuous-flow reactor regime, durations of light and
372 dark phases in a diel cycle set the availability of substrate and the achievement of a steady
373 state. Longer dark phases result in an excess of substrate available in the light phase for
374 biomass growth. Longer light phases lead to substrate limitation and steady conditions.
- 375 2) Even in actively growing cells, carbon allocation is in place, namely toward PHA
376 production.
- 377 3) In growing cells, H₂ production during illumination is a minor electron sink and secondary
378 to PHA production.

379

380 **Material and Methods**

381 **Strain**

382 The *Rhodopseudomonas* strain was isolated with dilution series in agar from an in-house PNSB
 383 enrichment culture designed for nutrient removal from synthetic wastewater (30). The isolate was
 384 characterized by full-length 16S rRNA gene sequencing. The genomic DNA was extracted using
 385 UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc., USA), following
 386 manufacturer's instructions. The full 16S rRNA gene was amplified with the primers forward U515
 387 (5'-GTGYCAGCMGCCGCGGTA-3') and reverse U1071 (5'-GARCTGRCGRCRRCCATGCA-
 388 3') (31), and sequenced for phylogenetic identification using a Sanger sequencing (Baseclear, NL).
 389 The sequence was aligned over the Blast database (32), and resulted in a 97.2% identity with
 390 *Rhodopseudomonas palustris*.

392 **Medium**

393 The inflow medium was adapted from Cerruti et al. (30). It consisted of (per liter): 0.914 g
 394 CH₃COONa·3H₂O (13.5 C-mmol L⁻¹ or 54 mmol electrons L⁻¹ when expressed via degree of
 395 reduction and 432 mg COD L⁻¹ when expressed as chemical oxygen demand), 0.229 g NH₄Cl (*i.e.*,
 396 4.281 mmol N L⁻¹ or 60 mg N-NH₄⁺ L⁻¹), 0.014 g KH₂PO₄ and 0.021 g K₂HPO₄ (*i.e.*, 0.223 mmol P
 397 L⁻¹ or 7 mg P L⁻¹), 0.200 g MgSO₄·7H₂O, 0.200 g NaCl, 0.050 g CaCl₂·2H₂O, 1 mL vitamins
 398 solution, 1 mL trace element solution, and 4.7 g 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 399 (HEPES) used as pH buffer.

400

401 The vitamin solution was composed of (per liter): 200 mg thiamine-HCl, 500 mg niacin, 300 mg p-
 402 amino-benzoic acid, 100 mg pyridoxine-HCl, 50 mg biotin and 50 mg vitamin B12.

403 The trace element solution was composed of (per liter): 1100 mg Na EDTA·2H₂O, 2000 mg
404 FeCl₃·6H₂O, 100 mg ZnCl₂, 64 mg MnSO₄·H₂O, 100 mg H₃BO₃, 100 mg CoCl₂·6H₂O, 24 mg
405 Na₂MoO₄·2H₂O, 16 mg CuSO₄·5H₂O, 10 mg NiCl₂·6H₂O and 5 mg NaSeO₃.

406

407 The medium components were sterilized though autoclavation or filtration with 0.22 µm filters
408 (Whatman, USA) (acetate solution, trace elements and vitamins).

409

410 **Reactor setup**

411 Continuous cultures were run to evaluate the influence of diel cycles on the physiology of PNSB. A
412 1.5-L continuous-flow stirred-tank reactor with 1.2-L working volume was connected to a
413 programmable logic controller (In-Control and Power unit, Applikon, NL) and operated under
414 stirring of 350 rpm, pH 7.0 ± 0.5, temperature of 30±1 °C during illumination phases and 20°C
415 during dark phases. Argon gas (Linde, NL, >99% purity) was sparged continuously in the bulk
416 liquid phase at 120 mL min⁻¹ to maintain anaerobic conditions. The continuous-flow rate was set at
417 0.048 L h⁻¹. It corresponded to a dilution rate of 0.04 h⁻¹ chosen based on the growth rate of the
418 strain previously measured at 0.11 h⁻¹ (data not shown). The biomass was maintained at a low
419 concentration of 0.26 ± 0.05 g DW L⁻¹ to minimize light shading effects.

420

421 The reactor was placed in a shaded hood to tightly control the irradiation patterns. Two halogen
422 floodlight lamps (Handson, NL) were positioned at opposite sides of the reactor diameter. The
423 incident white light spectrum was filtered for infrared (IR) light ($\lambda > 700$ nm) using two filter sheets
424 of 70 x 70 cm (Black Perspex 962, Plasticstockist, UK). The IR light intensity measured at the
425 reactor surface with a pyranometer (CMP3; Kipp & Zonen, NL) was 300 W m⁻². An automatic
426 device was used to switch on/off the light at required time sets. Three light conditions were tested:
427 1) continuous illumination (*i.e.*, 24 h light), 2) 16 h light and 8 h dark cycles, and 3) 8 h light and 16
428 h dark cycles.

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Analytical methods

The biomass concentration was measured by absorbance at 660 nm (A_{660}) using a spectrophotometer (Biochrom, Libra S11, USA). A calibration curve was established to correlate A_{660} to dry weight (DW) concentration: $c(\text{g DW L}^{-1}) = 0.64 A_{660} - 0.06$. Biomass dry weight was measured taking samples from the liquid phase, filtering them using 0.45 μm filters (Whatman, USA) and storing them in a 70 °C stove for 72 h (adapted from Lip et al. 2020).

Acetate was measured with a high-performance liquid chromatograph (HPLC) (Waters, 2707, NL) equipped with an Aminex HPX-87H column (BioRad, USA). Samples were eluted using H_3PO_4 (1.5 mmol L^{-1} , flowrate of 0.6 mL min^{-1} , temperature of 60°C) prior to refraction index (Waters 2414) and UV (210 nm, Waters 484) detections.

Ammonium (as N-NH_4^+) and orthophosphate (as P-PO_4^{3-}) concentrations were measured with a discrete analyser (Thermoscientific Gallery, NL).

PHA measurements were performed with fluorimetry for high sensitivity on small biomass samples. The low biomass concentration did not allow for traditional extractions of PHA and gas chromatographic measurements of monomers. PHAs were stained in the biological samples with Nile red (CAS n. 7385-67-3, Sigma Aldrich), as in (34). Fluorescence was measured with a microplate reader (1000M pro, Tecan), with excitation at 535 nm and emission at 605 nm. The fluorescent counts were normalized by the g DW of biomass present in analysed samples. The absorbance at 660 nm was measured in each well, and then converted in gDW biomass, to this end.

The CO₂ and H₂ in the offgas were measured using a mass spectrometer (ThermoFisher, Prima BT Benchtop MS) connected online to the bioreactor. The production rates of these components were calculated using the argon gas inflow rate (120 mL h⁻¹).

Mathematical model

A simple mathematical model was developed to characterize the behavior of the continuous culture of *Rhodospseudomonas* exposed to light/dark cycles. Under light, photoorganoheterotrophic growth was assumed; under dark, completely ceased metabolic activity. The growth stoichiometry with light as energy source followed equation (1), with the biomass composition adapted from (35):

The process was described by a system of balance equations for the relevant materials in the mixed liquor (eqns. (2) (biomass C_X , acetic acid C_S , ammonium C_N , phosphate C_P and carbon dioxide C_C).

$$\frac{dC_X}{dt} = \frac{F}{V} \cdot (-C_X) + r_X, \quad \frac{dC_C}{dt} = \frac{F}{V} \cdot (-C_C) + r_C + k_L a \cdot (C_C^* - C_C) \quad (2)$$

$$\frac{dC_S}{dt} = \frac{F}{V} \cdot (C_{S,in} - C_S) + r_S, \quad \frac{dC_N}{dt} = \frac{F}{V} \cdot (C_{N,in} - C_N) + r_N, \quad \frac{dC_P}{dt} = \frac{F}{V} \cdot (C_{P,in} - C_P) + r_P$$

A gas phase balance was integrated for the CO₂ concentration in the gas $C_{C,g}$ (mmol/L):

$$\frac{dC_{C,g}}{dt} = -\frac{F_g}{V_g} C_{C,g} - k_L a \cdot (C_C^* - C_C) \cdot \frac{V}{V_g} \quad (3)$$

The volumetric growth rate, r_X (C-mmol X L⁻¹ h⁻¹), was assumed to be limited by the concentrations of multiple chemical compounds and by light intensity I (W m⁻²):

$$r_X = \mu_{max} \cdot \frac{C_S}{K_S + C_S} \cdot \frac{C_N}{K_N + C_N} \cdot \frac{C_P}{K_P + C_P} \cdot \frac{I}{K_I + I} \cdot C_X \quad (4)$$

The uptake and production rates of chemical compounds ($\text{mmol L}^{-1} \text{ h}^{-1}$) follow from the reaction stoichiometry and biomass growth rate:

$$r_S = -\frac{1}{Y_{XS}} \cdot r_X, \quad r_N = -Y_{NX} \cdot r_X, \quad r_P = -Y_{PX} \cdot r_X, \quad r_{CO_2} = Y_{CX} \cdot r_X$$

Similarly, the biomass-specific rates were calculated as $q_S = \mu / Y_{XS}$, $q_N = Y_{NX} \mu$, $q_P = Y_{PX} \mu$ and $q_C = Y_{CX} \mu$. The yields Y_{XS} , Y_{CX} and max. specific growth rate μ_{max} were determined by fitting the measurements from the 16h light / 8h dark experiment with this model. Additional estimations of the μ_{max} based on batches and chemostat experiments under continuous illumination and light / dark patterns resulted in μ_{max} values of 0.14 ± 0.05 . The yields Y_{NX} and Y_{PX} (and consequently the N and P biomass composition nN and nP , respectively) were determined from the 8h light / 16h dark experiment, for which we were able to record better quality data.

The liquid flow rate F (L h^{-1}) and volume V (L), the gas flow rate F_g (L h^{-1}) and volume V_g (L), as well as the concentrations of chemical compounds in the influent C_{in} (mmol L^{-1}) were all fixed in the experiments. The volumetric mass transfer coefficient k_{La} (h^{-1}) was determined from the correlation with the power input, liquid volume, and gas velocity (see SI-1). The dissolved CO_2 concentration, C_c^* (mmol L^{-1}), was calculated function of gas concentration $C_{c,g}$, Henry coefficient H_C ($\text{mol L}^{-1} \text{ atm}^{-1}$) and respective temperature (20°C in dark and 30°C in light) and pressure (1 atm).

Two light sources with intensity I_0 were placed at opposite sides next to the reactor. The light intensity decrease away from the reactor wall was assumed to follow the Beer-Lambert attenuation

497 law. Therefore, by summing the light intensities coming from both sides one obtains the total light
498 intensity I_t at a radial position x (m) in the reactor:

$$499 \quad I_t = I_0 \cdot \left(e^{-\varepsilon \cdot c_X \cdot x} + e^{-\varepsilon \cdot c_X \cdot (2 \cdot R - x)} \right) \quad (5)$$

500 with R (m) the reactor radius, ε ($\text{m}^2 \text{g}^{-1}$) the biomass-specific light attenuation coefficient and C_X
501 (g m^{-3}) the biomass concentration. We considered that due to liquid mixing the cells will be exposed
502 to an average light intensity I (W m^{-2}) computed by integrating eq. (5) over the reactor diameter:

$$503 \quad I = I_0 \cdot \frac{1 - e^{-2 \cdot \varepsilon \cdot c_X \cdot R}}{\varepsilon \cdot c_X \cdot R} \quad (6)$$

504 The model was solved in MATLAB (R2018b, Mathworks, Natick, MA, www.mathworks.com)
505 using a stiff solver for the ordinary differential equations system (2) and (3). The initial
506 concentrations were taken to coincide with the measurements. The parameter estimation was
507 performed by a constrained optimization routine minimizing the sum of squares of relative errors
508 between model and experimental data. The fixed model parameters and the fitted parameters are
509 listed in Table 1. A more extensive description of the parameter used is available in SI-1.

510

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517

518 **Conflict of interest statement**

519 The authors declare no conflict of interest.

520

521 **Preprint**

522 This manuscript will be deposited as pre-print in bioRxiv.

523

524 **Supplementary information**

525 Appendix 1: Estimation of the kLa of the process

526 Appendixes 2: Mathematical model simulations

- 527 • Fig. A-1: Data and simulation of the 8 h light / 16 h dark cycles with extended light phase.
- 528 • Fig. A-2: Model results for the light intensity inside the reactor
- 529 • Fig. A-3: Model results for production and uptake rate in the 16 h light / 8 h dark cycles
- 530 • Fig. A-4: Model results for production and uptake rate in the 8 h light / 16 h dark cycles.

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709 **Figure legends**

710 **Figure 1.** Schematic representation of the reducing power allocation in *Rhodopseudomonas*. Red
711 lines: electron flow; Black dashed lines: proton transfer; Blue dotted lines: NADH is normally
712 produced in the catabolic processes, whereas NADPH is used in the anabolic processes, such as
713 PHAs formation. Biomass and CBB are the primary electron sinks in PNSB. PHAs and H₂ are two
714 of the other possible electron sinks.

715 **Figure 2.** Dynamics of biomass, acetate, ammonium and phosphate concentrations during light-
716 dark cycles: (A),(C) 16 h light / 8 h dark; (B),(D) 8 h light / 16 h dark. Gray areas represent the
717 dark periods. (A),(B) biomass (black circles) and acetate (red open squares) measured
718 concentrations, with lines being the model results. The biomass concentration achieved a stationary
719 state, while acetate reached very low (limiting) concentrations after 2 h of the 16 h light periods.
720 The biomass increased without reaching a steady state during the 8 h light periods, while acetate
721 was not fully consumed. (C),(D) ammonium (blue diamonds) and phosphate (green triangles)
722 measured concentrations, with lines being the model results. Concentrations of N and P were not
723 limiting for the system in any illumination phase.

724 **Figure 3.** Dynamics of CO₂, H₂ and PHA during light-dark cycles: (A),(D) continuous illumination;
725 (B),(E),(G) 16 h light / 8 h dark; (C),(F),(H) 8 h light / 16 h dark. Gray areas represent the dark
726 periods. (A),(B),(C) CO₂ production rate - measured (red circles) and computed (lines). CO₂
727 production was constant during continuous illumination, but peaks appeared during light-dark
728 cycles, correlated with the acetate uptake. (D),(E),(F) measured H₂ production (blue circles).
729 Constant low level H₂ was produced during continuous illumination and 16 h light cycles, but H₂
730 production strongly increased in each light period of the 8 h light cycles. (G),(H) PHAs fluorescent
731 counts per gram biomass in the light/dark experiments. A PHAs peak was measured at the
732 beginning of each light phase under the 16 h light conditions, however less clear pattern could be
733 detected under the 8 h light experiments.

Tables

Table 1. Model parameters. The maximum biomass-specific growth rate and yields were fitted to experimental data, while the other parameters were measured during experiments, calculated or obtained from literature.

Parameter name	Symbol	Value	Units	Source
Max. biomass-specific growth rate	$\mu_{\max}$	0.15	$\text{C-mol X} \cdot \text{h}^{-1} \cdot \text{C-mol}^{-1} \text{X}$	Fitted
Biomass yield	Y_{XS}	0.88	$\text{C-mol X} \cdot \text{C-mol}^{-1} \text{S}$	Fitted
Ammonium yield	Y_{NX}	0.18	$\text{mol} \cdot \text{C-mol}^{-1} \text{X}$	Fitted
Phosphate yield	Y_{PX}	0.009	$\text{mol} \cdot \text{C-mol}^{-1} \text{X}$	Fitted
CO₂ yield	Y_{CX}	0.142	$\text{mol} \cdot \text{C-mol}^{-1} \text{X}$	Fitted
Reactor radius	R	0.055	m	Measured
Liquid volume	V	1.2	L	Measured
Liquid flow rate	F	0.048	$\text{L} \cdot \text{h}^{-1}$	Measured
Dilution rate	D	0.04	h^{-1}	F/V
Gas volume	V_g	0.3	L	Measured
Gas flow rate	F_g	7.14	$\text{L} \cdot \text{h}^{-1}$	Measured
Inflow acetate concentration	$C_{S,in}$	7	mM	Measured
Inflow ammonium concentration	$C_{N,in}$	4.28	mM	Measured
Inflow phosphate concentration	$C_{P,in}$	0.22	mM	Measured
Light intensity at reactor walls	I_0	300	$\text{W} \cdot \text{m}^{-2}$	Measured
Light extinction coefficient per biomass concentration	ε	0.1	$\text{m}^2 \cdot \text{g}^{-1}$	(17)
Half-saturation coefficient acetate	K_S	0.1	mM	Lower than in (36) (0.3 mM)
Half-saturation coefficient ammonium	K_N	0.001	mM	(37)
Half-saturation coefficient phosphate	K_P	0.003	mM	(38)
Half-saturation coefficient light	K_I	10	$\text{W} \cdot \text{m}^{-2}$	(39)
CO₂ mass transfer coefficient	k_{La}	32	h^{-1}	Calculated (SI-1)
Henry coefficient CO₂	H_C	0.03 (at 30°C) 0.04 (at 20°C)	$\text{mol} \cdot \text{L}^{-1} \cdot \text{atm}^{-1}$	(40)

739 **Table 2.** Distribution of the carbon sources and carbon balances in different illumination conditions

Light/dark regimes	Carbon inputs		Carbon outputs			Carbon balance	Electron balance
	(C-mmol h ⁻¹)		(C-mmol h ⁻¹)			(% C)	(% COD)
Component	Acetate	Acetate	Biomass	CO ₂	Σ out		
Phase	Influent	Effluent	Effluent	Off-gas			
24 h light	0.66±0.05	<LOD ^a	0.58	0.07±0.001	0.65	98%	98%
16 h light	0.66±0.05	0.11±0.01	0.57±0.01	0.08±0.01	0.75±0.01	115±2%	113±1%
8 h dark		0.10±0.02	0.51±0.03	0.07±0.02	0.68±0.06	103±9%	102±9%
8 h light	0.61±0.006	0.28±0.03	0.62±0.05	0.07±0.01	0.97±0.05	158±8% ^b	159±9% ^b
16 h dark		0.19±0.07	0.57±0.12	0.03±0.002	0.79±0.15	130±25% ^b	137±27% ^b

740 ^a limit of detection; ^b the overvaluation of the carbon and electron balance can be due to an overestimation of the biomass concentration. The
741 absorbance measures taken might have influenced by the presence of PHAs inside the cells.

742

Supplementary information

Appendix 1: Estimation of the kLa 's of the process

The overall mass transfer coefficients of the process were found in the CO_2 and O_2 mass balances. The calculation of the oxygen mass transfer coefficient was performed considering the equation for salt solutions

$$kLa \text{ (s}^{-1}\text{)} = 0.002 \cdot \left(\frac{P}{V_{liquid}} \right)^{0.7} \cdot u_{gas}^{0.4}$$

where P is the power input (W), u_{gas} is the superficial gas velocity (m/s) and V_{liquid} the liquid volume (m^3). The power input was calculated through the following equation (60)

$$P(W) = \rho \cdot N_p \cdot N_i^3 D_i^5$$

where ρ is the density ($kg \text{ m}^{-3}$) of the solution, N_p is the impeller power number, N_i is the impeller rotating speed (s^{-1}) and D_i is the impeller diameter (m).

The power number of the impeller is a function of the impeller type and the Reynolds number of the impeller. Reynolds was calculated through the following equation

$$Re = \frac{\rho N_i D_i^2}{\mu}$$

where μ is the viscosity of the solvent ($Pa \cdot s$). Considering the density (997 kg m^{-3}) and viscosity of water in $25^\circ C$ ($0.00089 \text{ Pa} \cdot s$) the Reynolds impeller was slightly higher than 10^5 . For that Reynolds and for a 6-blade Rushton turbine impeller the power number is around 5~6 (60). For the calculations, a power number of 6 was assumed. The diameter of the impeller was around 4 cm and the rotating speed 350 rpm (5.83 s^{-1}). Using those values the power input of the impeller was found equal to 0.101 W.

768 The superficial gas velocity was calculated by dividing the gas flowrate (7.14 L h⁻¹) with the surface
 769 area (πR^2). The reactor radius R was 5.5 cm. After dividing and correcting for the units the
 770 calculated superficial gas velocity was 0.000209 m s⁻¹. Using the calculated values of the superficial
 771 gas velocity, power input and liquid volume (1.2 · 10⁻³ m³) the k_La of oxygen was found equal to
 772 33.52 (h⁻¹).
 773 The k_La of carbon dioxide was found equal to 32 (h⁻¹) through the following equation (61). The
 774 diffusion coefficient used are 1.92 · 10⁻⁵ cm² s⁻¹ (62)

775

$$776 \quad kLa_x = \frac{kLa_{CO_2}}{\sqrt{\frac{D_{CO_2}}{D_x}}}$$

777

Appendix 2 to 5: Mathematical model simulations

Figure A-2. Data and simulation with an additional extended light phase after the 8 h light 16 h dark cycles. (A) Biomass (black circles) and acetate (red open squares) measured concentrations, with lines being the model results. (B) Measured (red circles) and model prediction (line) of the CO_2 production rate. A steady state is reached once the concentration of acetate is at its minimum.

Figure A-3. Model results for the light intensity inside the reactor. (A) 16 h light / 8 h dark cycles. (B) 8 h light / 16 h dark cycles. Under any given condition light became a limiting factor.

Figure A-4. Model results for production and uptake rate in the 16 h light / 8 h dark cycles. (A) specific growth rate. (B) specific acetate uptake rate. (C) specific ammonium uptake rate. (D) specific phosphate uptake rate. (E) specific CO₂ production rate.

Figure A-5. Model results for production and uptake rate in the 8 h light / 16 h dark cycles. (A) specific growth rate. (B) specific acetate uptake rate. (C) specific ammonium uptake rate. (D) specific phosphate uptake rate. (E) specific CO₂ production rate.

Figure 1. Schematic representation of the reducing power allocation in *Rhodospseudomonas*. Red lines: electron flow; Black dashed lines: proton transfer; Blue dotted lines: NADH is normally produced in the catabolic processes, whereas NADPH is used in the anabolic processes, such as PHAs formation. Biomass and CBB are the primary electron sinks in PNSB. PHAs and H₂ are two of the other possible electron sinks.

Figure 2. Dynamics of biomass, acetate, ammonium and phosphate concentrations during light-dark cycles: (A),(C) 16 h light / 8 h dark; (B),(D) 8 h light / 16 h dark. Gray areas represent the dark periods. (A),(B) biomass (black circles) and acetate (red open squares) measured concentrations, with lines being the model results. The biomass concentration achieved a stationary state, while acetate reached very low (limiting) concentrations after 2 h of the 16 h light periods. The biomass increased without reaching a steady state during the 8 h light periods, while acetate was not fully consumed. (C),(D) ammonium (blue diamonds) and phosphate (green triangles) measured concentrations, with lines being the model results. Concentrations of N and P were not limiting for the system in any illumination phase.

Figure 3. Dynamics of CO₂, H₂ and PHA during light-dark cycles: (A),(D) continuous illumination; (B),(E),(G) 16 h light / 8 h dark; (C),(F),(H) 8 h light / 16 h dark. Gray areas represent the dark periods. (A),(B),(C) CO₂ production rate - measured (red circles) and computed (lines). CO₂ production was constant during continuous illumination, but peaks appeared during light-dark cycles, correlated with the acetate uptake. (D),(E),(F) measured H₂ production (blue circles). Constant low level H₂ was produced during continuous illumination and 16 h light cycles, but H₂ production strongly increased in each light period of the 8 h light cycles. (G),(H) PHAs fluorescent counts per gram biomass in the light/dark experiments. A PHAs peak was measured at the beginning of each light phase under the 16 h light conditions, however less clear pattern could be detected under the 8 h light experiments.