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

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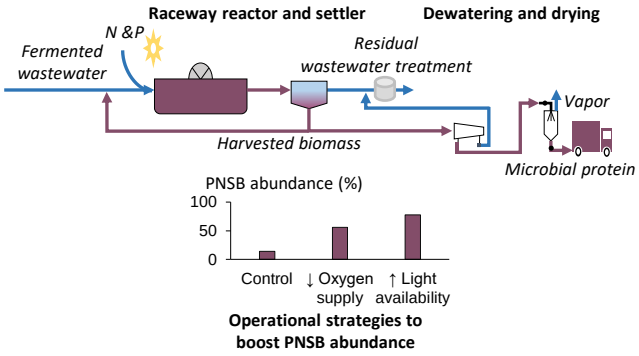
Graphical abstract



Abstract

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

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

1 Introduction

Inefficiencies in the fertilizer-food chain severely distort the carrying capacity of the Earth, surpassing the planetary boundaries (i.e. safe operating space for sustainability) beyond the zone of uncertainty.[1] Mitigation can be brought about by upgrading wastewater resources to microbial protein or single-cell protein, which is the use of microorganisms as an ingredient in animal feed or human food.[2,3] Protein production from industrial wastewater installations with separated process water and sanitary water is preferred, as European regulation No 767/2009 prohibits the use of waste obtained from feces and urine as feed material.[4] Muys, et al. 2020[5] performed a screening of 19 wastewater installations in the food and beverage industry and observed that 6 companies already separated process water and sanitary wastewater. The study of Muys, et al. 2020[5] also showed that brewery and dairy wastewater is particularly interesting for protein production as heavy metals in the activated sludge were below legal limits for feed ingredients.

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

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

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

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

Raceway reactors are easy to operate, maintain and clean.[24] These reactors are open systems with a longer light path (e.g. liquid depth of 20 cm vs. 6 cm diameter tubular PBR), a lower lit surface-to-volume ratio of 5 m^2 m^{-3} (vs. 22 m^2 m^{-3} tubular PBR) and are agitated with a paddlewheel (vs. circulation pumps in tubular PBR).[25] In the context of wastewater treatment, another function is envisaged for PNSB compared to microalgal raceway systems. More specifically, the loading rate, SRT and treatment stage differ. For microalgae, raceway reactors are used for both secondary and tertiary treatment operated at low loading rates (81-388 mg COD L^{-1} d^{-1}) and long SRT (4-8 d; 31°C).[26,27] Raceway systems for PNSB, on the other hand, would mainly be used for secondary treatment at higher loading rates and shorter SRT.

Raceway reactor could be economically interesting for PNSB yet, achieving selective production is more challenging in these reactors, as air continuously enters the system, which enables the proliferation of competing aerobic heterotrophs (i.e. non-PNSB). Moreover, the oxygen concentration in a raceway reactor is zero due to its direct use as an electron acceptor, making the growth of anaerobic chemotrophs such as acidogenic microorganisms and sulfate-reducing bacteria (SRB) also possible. According to the authors' knowledge, research on PNSB production in raceway reactors is limited. One conference abstract by Fradinho, et al. 2019[28] focuses on polyhydroxyalkanoate production with PNSB in raceway reactors, yet specific operational conditions are not fully described. It can, however, be hypothesized that the

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

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

2 Materials and methods

2.1 Inocula and medium

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

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

A synthetic medium was chosen over real wastewater to avoid inherent variability from real wastewater and to clearly discriminate the effect of specific operational strategies on the microbial community. Volatile fatty acids (VFA) were used as carbon source as a proxy for fermented wastewater, as we argued in a previous study that fermentation prior to protein production will favor the microbial selectivity.[2,12] The COD concentration was 3 g L^{-1} and contained a defined mixture of acetate, propionate and butyrate on a 1/1/1 COD basis. The medium also contained 0.8 g L^{-1} KH_2PO_4, 0.5 g L^{-1} $MgCl_2.6H_2O$, 0.1 g L^{-1} $CaCl_2.2H_2O$, 0.7 g L^{-1} Na_2SO_4, 1.2 g L^{-1} NH_4Cl, 1.0 g L^{-1} NaCl and 0.3 g L^{-1} $NaHCO_3$. 1 mL of trace elements and 1 mL of vitamin solution, based on the composition of Imhoff 2006[10], was also added per liter of water. PNSB grown photoheterotrophically on this VFA mixture have a biomass yield that approximate 1 g $COD_{biomass}$ g^{-1} $COD_{removed}$.[12,29] This makes it easy to assess the chemoheterotrophic growth of PNSB and of competing non-PNSB, as a lower biomass yield implies oxidation of COD to CO_2. Note that the overall biomass yield in a treatment system will be lower than 1 as COD removed during the anaerobic fermentation will also be included.

2.2 Overview of the experiments

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

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

Objective	**DO** (mg O_2 L^{-1})	**OTR** (mg O_2 L^{-1} d^{-1})	**Stirring** (on/off)	**Illumination** (light/dark)	**S/V ratio** (m^2 m^{-3})	**HRT** (d)	**Inoculum**	**Cultivation**	**Reactor type**
Assess metabolic growth modes of PNSB and non-PNSB (section 3.1)	N.D.*	331**	24h/0h	24h	61***	Batch ****	PNSB	Axenic	Flask
	-	0		24h			PNSB		
	N.D.*	331**		0h			PNSB		
	-	0		0h			PNSB		
	N.D.*	331**		0h			Non-PNSB	Non-axenic	
	-	0		0h			Non-PNSB		
Effect of SRT on PNSB growth (section 3.2)	0.18 ± 0.04	331	24h/0h	12h/12h	61	1.25	PNSB	Non-axenic	Flask
	0.18 ± 0.06	331				2			
	0.15 ± 0.04	333				3			
PNSB selectivity over sequential batches (section 3.3)	0.17 ± 0.15	219	24h/0h	12h/12h	5***	2	PNSB	Non-axenic	Raceway
	0.14 ± 0.12	220	12h/12h		5	2			
	0.24 ± 0.12	217	24h/0h		10	2			

*DO was not determined (N.D.) during the axenic experiments as it would result in contamination.
**The DO concentration for the OTR calculation was assumed to be 0.18 mg O_2 L^{-1}.
***The surface area exposed to air for the flask experiments was equal to 11 m^2 m^{-3} and for the raceway reactor 5-10 m^2 m^{-3}.
****Growth experiment between 50-150h, stopped when the stationary phase was reached

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

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

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

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

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

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

Experiments were performed under combined photo- and chemotrophic conditions, allowing the entry of oxygen along with illumination (i.e. conditions prevalent in raceway reactor). Flasks of 500 mL were used as reactors and illuminated through a natural 12-h light/12-h dark regime with two halogen lamps at a light intensity of 30 W m^{-2} (vs. previous flask experiments section 2.2.1: 24-h light or 24-h dark). The flasks were filled with 200 mL of medium (COD concentration 3 g L^{-1} see section 2.1) corresponding to a maximum OTR of 336 mg O_2 L^{-1} d^{-1}. An influent COD concentration of 6 g L^{-1} was also tested to study the effect of a higher COD-loading rate on the microbial selectivity (more COD available during the night). The experiment was performed non-axenically with *Rb. capsulatus* as initial inoculum. The tested SRT were 1.25 d, 2 d and 3 d. The hydraulic retention time was equal to the SRT. All tests were performed in biological duplicate. A detailed description of the methodology can be found in Supporting Information S4.

2.2.3 Operational strategies to steer PNSB selectivity and reactor performance

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

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

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

2.3 Analytical procedures

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

2.4 Microbial community analyses

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

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

2.5 Statistical analyses

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

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

3 Results and discussion

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

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

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

assimilation of COD than oxidation to CO_2. The bacteriochlorophyll content was lower for the combined photo- and chemotrophic metabolism than for the phototrophic metabolism, yet still 6 times higher than for the aerobic chemotrophic growth (i.e. no illumination with oxygen supply). It should be noted that the chemotrophic metabolism of PNSB contributes more to growth when the oxygen supply increases. Supporting Information S3 shows that growth rates for the combined photo- and chemotrophic metabolism do not change, yet the aerobic chemotrophic growth increases. This implies that chemotrophic growth contributed more to the combined photo- and chemotrophic metabolism at a higher oxygen supply. It remains, however, unclear whether the same PNSB species performs both metabolisms simultaneously or if there is a division of labor between bacteria. More research is needed to elucidate this.

In a full-scale raceway reactor, the DO concentration will approximate zero due to the direct consumption of oxygen, allowing anaerobic fermentation of COD. The anaerobic chemotrophic growth of PNSB was, therefore, tested with a more complex medium of 3.7 g COD L^{-1} containing a mixture of organics such as yeast, peptone, malt extract, acetate, propionate and butyrate (details Supporting Information S1). PNSB were able to anaerobically ferment organics (Figure 1). Growth rates and biomass yields were respectively 0.3 ± 0.08 d^{-1} and 0.16 ± 0.09 g $COD_{biomass}$ g^{-1} $COD_{removed}$, lower than their combined photo- and chemotrophic growth (1.75 ± 0.05 d^{-1}; 0.71 ± 0.07 g $COD_{biomass}$ g^{-1} $COD_{removed}$). A similar observation was made by Schultz Weaver 1982[33] with low anaerobic growth rates and biomass yields for *Rb. capsulatus* (≈ 0.08 d^{-1}; 0.09 g $COD_{biomass}$ g^{-1} $COD_{removed}$) and *Rhodospirillum rubrum* (≈ 0.13 d^{-1}; 0.11 g $COD_{biomass}$ g^{-1} $COD_{removed}$). The non-PNSB inoculum showed growth rates of 0.58 ± 0.03 d^{-1} or 2 times higher compared to PNSB. Therefore, anaerobic fermentation will mainly be performed by competing non-PNSB, which can act as symbionts by making COD available for PNSB. Stronger competition during the light and dark period might arise from aerobic chemotrophic non-PNSB since their growth rate was 2.8 times higher than for the

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

PNSB cells were able to produce bacteriochlorophyll a during the aerobic and anaerobic batch test in the dark and in the light (Figure 1A) because the activation of photosynthetic genes is dependent on the oxidative conditions.[34] Ghosh, et al. 1994[35], for example, found that the formation of photosynthetic membranes is triggered at DO concentrations lower than 0.40 mg O_2 L^{-1}.

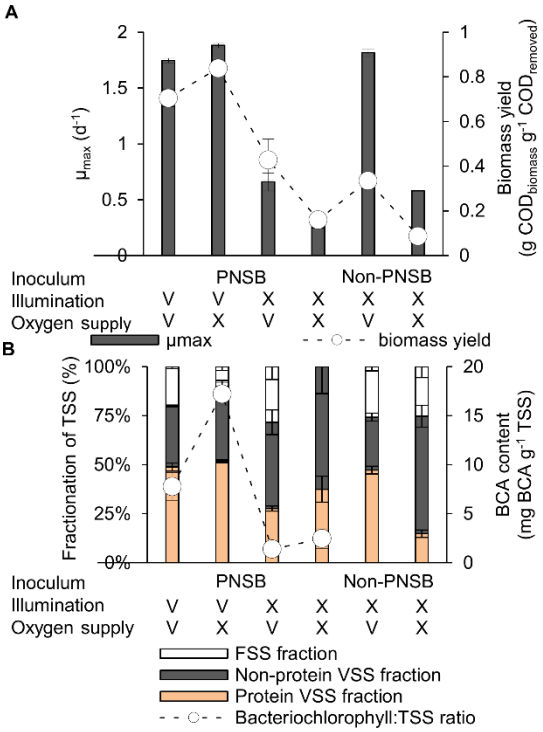


Figure 1 (A) maximum specific growth rate and biomass yield for purple non-sulfur bacteria (PNSB) and non-PNSB along with (B) biomass fractionation and bacteriochlorophyll a (BCA) content. Tested conditions: combined photo- and chemoheterotrophic (illumination: V; oxygen supply: V), photoheterotrophic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

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

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

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

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

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

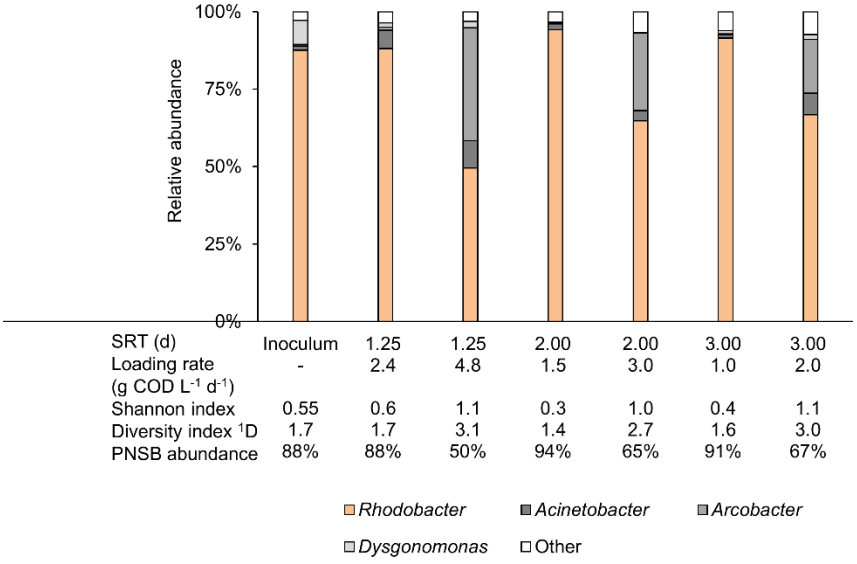


Figure 2 Effect of sludge retention time (SRT) on microbial community composition, Shannon's H index, exp(H') and purple non-sulfur bacteria (PNSB) abundance. Flasks were used as a reactor. The PNSB genera *Rhodobacter* and *Rhodopseudomonas* are all marked in orange. Samples obtained after 2-10 SRT.

3.3 Reactor performance and community dynamics over sequential batches

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

The highest productivities (0.21 g protein L^{-1} d^{-1} corresponding with 0.43 g TSS L^{-1} d^{-1}; Figure 3) and removal rates (0.79 g COD L^{-1} d^{-1}) were achieved when PNSB were most abundant (Figure 4). This was during the experimental condition of 24-h stirring and 12-h light/12-h dark at the highest surface-to-volume ratio of 10 m^2 m^{-3}. A higher ratio of 10 m^2 m^{-3} increased the light availability, resulting in higher biomass concentrations (0.81 ± 0.04 g TSS L^{-1}) relative to the benchmark of 5 m^2 m^{-3} (0.62 ± 0.02 g TSS L^{-1}). For a closed PBR operated on the same medium at an SRT of 1 d (vs. 2 d for raceway reactor), we reached TSS productivities that were 1.5-2.6 times higher compared to the raceway reactor.[12] This was probably due to the higher light availability in the PBR compared to a raceway reactor (surface-to-volume ratio 33 vs. 5-10 m^2 m^{-3}). Capson-Tojo, et al. 2020[19] have compared the biomass productivity of several PNSB reactors. The productivity for closed PBR were maximally 0.77 g TSS L^{-1} d^{-1} (10-39°C), 1.8 times higher than the productivity in this study (28°C). For the COD removal rate, values between 0.45-0.79 g $COD_{removed}$ L^{-1} d^{-1} were reached in the raceway reactor, comparable with the median productivity of photo anaerobic membrane bioreactors (0.96 g $COD_{removed}$ L^{-1} d^{-1}. 10-39°C). For the microalga *Chlorella vulgaris* cultivated in the same reactor (12-h light/12-h dark), the productivity was 0.009 g protein L^{-1} d^{-1} or 22 times lower compared to PNSB.[36] This was probably due to the higher growth rates of PNSB of 0.6-

352 3.7 d^{-1} compared to the ones of microalgae of 0.60-1.38 d^{-1} (28°C).[12,37,38] This is also true for
353 microalgal bacterial floc technology, a wastewater treatment system where microalgae supply
354 oxygen to aerobic bacteria for COD removal.[27] Van Den Hende, et al. 2014[26] observed biomass
355 productivities and COD removal rates of respectively 0.07-0.22 g TSS L^{-1} d^{-1} and 0.06-0.30 g
356 $COD_{removed}$ L^{-1} d^{-1} with the microalgal bacterial floc technology (31°C), lower than the PNSB
357 raceway reactor operated in this study (0.24-0.43 g TSS L^{-1} d^{-1} and 0.45-0.79 g $COD_{removed}$ L^{-1}
358 d^{-1}; 28°C). The aerobic bacteria in the microbial community could theoretically cope with low
359 SRT, yet the system is operated at an SRT of 4-8 d to maintain microalgae in the reactor (vs. 2
360 d SRT in this study).

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

367 The bacteriochlorophyll a content of the biomass was between 6-10 mg g^{-1} TSS (Figure
368 3). *Rb. capsulatus* grown anaerobically in the light typically has values around 21-50 mg g^{-1}
369 TSS.[39] The lower pigment content in the raceway reactor compared to strict anaerobic systems
370 was due to aerobic chemoheterotrophic growth of PNSB. Ghosh, et al. 1994[35] have shown that
371 PNSB grown heterotrophically with oxygen can produce pigments at DO concentrations lower
372 than 0.40 mg O_2 L^{-1}. We observed that the bacteriochlorophyll a content of *Rb. capsulatus* was
373 1.4 mg g^{-1} TSS or 12 times lower compared to their anaerobic photoheterotrophic growth
374 (Figure 1A). Low pigmentation was also visually observed for aerobically grown *Rb.*
375 *sphaeroides*, *Rhodopseudomonas palustris* and *Rhodospirillum rubrum* in our previous
376 study.[13] It was, thus, likely that the overall bacteriochlorophyll a content of the biomass

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

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

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

Other PNSB genera were also present in the system such as *Rhodopseudomonas* (2-3%), and *Blastochloris* (< 0.2%). The main competing genera were *Acinetobacter* (aerobic chemoheterotroph), *Dysgonomonas* (anaerobic chemoheterotrophs), *Arcobacter* ((an)aerobic chemoheterotrophs) and *Alcaligenes* (aerobic chemoheterotrophs) with an abundance of respectively 1-4%, 1-12%, 0-31% and 1-54%. Microalgae are undesirable due to oxygen production and stimulation of aerobic chemotrophic COD conversion. These microbes were not detected through the absorbance spectra (no chlorophyll peaks) and no cyanobacteria were identified by amplicon sequencing. This was probably due to the short SRT (2 d; 28°C), resulting in washout of slower-growing microalgae (μ_{max} 0.60-1.38 d^{-1}; 28°C).[37] It could, therefore, be argued that SRT control and potentially additional measures in a real system are crucial to prevent microalgal growth. Although the sulfate concentration in the medium was 1.2 g L^{-1}, there were no SRB detected (Figure 4) and no sulfate was removed (Supporting Information S12). SRB require 0.7 g COD to remove 1 g of sulfate.[40] Therefore, they can contribute to COD removal, yet biomass production will be negligible due to their low biomass yields of 0.015-0.033 g VSS g^{-1} SO_4^{2-}.[41] An anaerobic fermenter is proposed prior to the raceway reactor, where sulfate can be converted to sulfide. The majority of PNSB grow only at levels less than 45 mg S L^{-1}, yet some species of *Rhodobacter* and *Rhodoferax* can tolerate sulfide concentrations up to 361 mg S L^{-1}.[42] In practice, sulfide will not be problematic as sulfate concentration, for example for brewery wastewater, are typical around 7 mg S L^{-1}.[43]

It is unlikely that biological N_2 fixation by PNSB occurred, as nitrogen concentrations were never limiting (effluent ammonium > 150 mg NH_4^+ N L^{-1}). Interestingly, a gap of around 91-124 mg NH_4^+ N L^{-1} exists in the nitrogen balance (Supporting Information S12 Table S3). This could in principle be due to nitrification and denitrification or through ammonia stripping.

Nitrification would theoretically be possible at an SRT of 2 days and a temperature of 28°C, yet no nitrifiers were detected in the microbial community (Figure 4) and the nitrite and nitrate concentration in the effluent was zero (Supporting Information S12 Table S3). At a pH of 7 and a temperature of 28°C, 7% of the total ammonia nitrogen is available as free ammonia. CO_2 sparging for pH control and stirring of the paddlewheel may have contributed to NH_3 stripping. Garcia, et al. 2000[44] for instance, observed that ammonia stripping accounted for 32-47% of the total nitrogen removal in an algae raceway reactor treating sewage (pH 8.6-9.4, temperature 12-27°C and HRT 3-10 d).

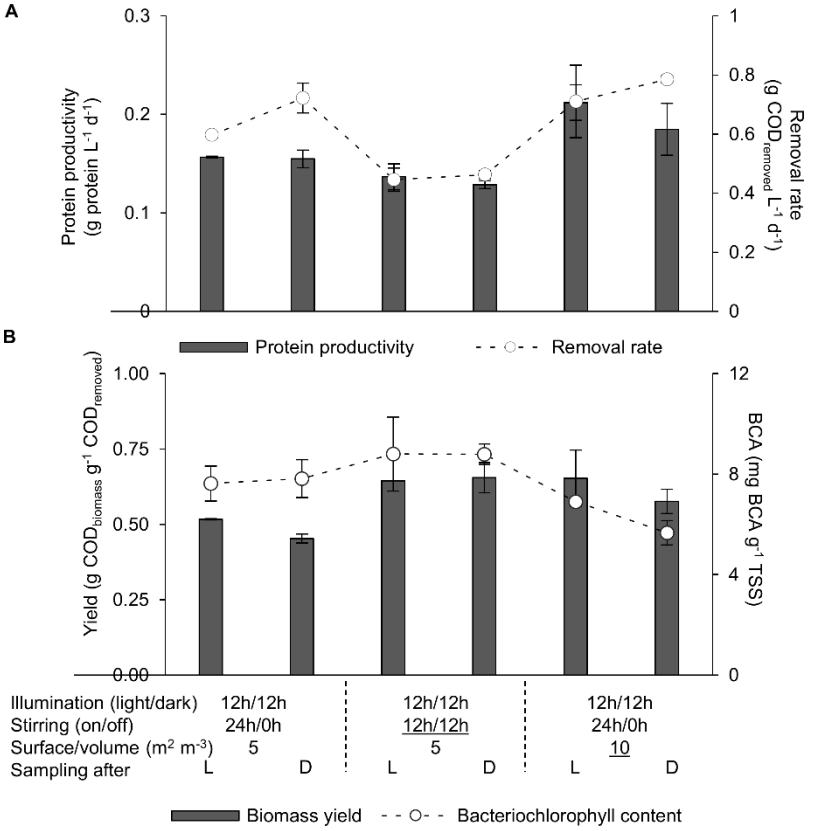


Figure 3 Production features of a raceway reactor operated at a sludge retention time of 2 d, testing the effect of oxygen (stirring) and light (surface-to-volume ratio). Sampling occurred after the light (L) or dark (D) period. Results show (A) the protein productivity and the volumetric removal rate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

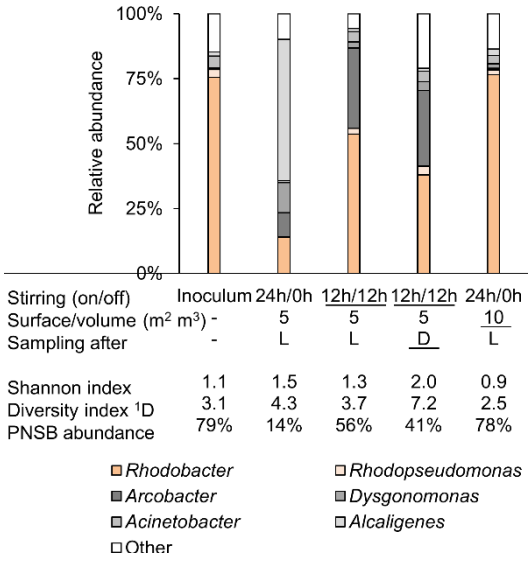


Figure 4 Raceway reactor operated at a sludge retention time of 2 d, testing the effect of oxygen (stirring) and the combination of light (surface-to-volume ratio). Sampling occurred after the light (L) or dark (D) period. operational strategy on microbial community composition, Shannon's H' index, exp(H')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

3.4 Challenges for upscaling a raceway reactor for PNSB production on wastewater

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

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

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

473 Another operational challenge for realistic wastewater treatment is the pH control system.
474 In the current set-up, CO_2 sparging was used, as is practiced in industrial microalgae cultivation
475 in raceway reactors where CO_2 delivery is essential to avoid carbon limitations.[46] For PNSB,
476 CO_2 only serves as an electron sink for more reduced electron donors such as propionate and
477 butyrate.[14] Acid dosage based on CO_2 sparging is, therefore, not essential for PNSB growth. In
478 the test set-up, the CO_2 sparging may unintentionally have created more favorable conditions
479 for PNSB by stripping out some dissolved oxygen (preventing additional aerobic conversions)
480 and sulfide (preventing potential toxicity). Follow-up research should also look into alternative
481 and economical pH control systems, for instance partially making use of the low influent pH
482 of fermentate.

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

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