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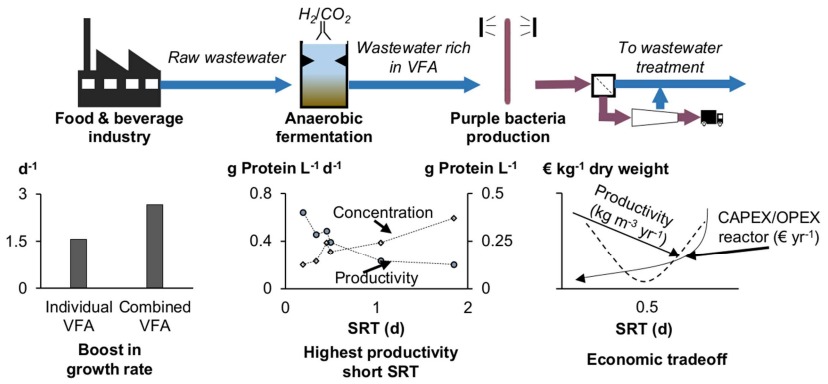

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Volatile fatty acids impacting phototrophic growth kinetics of purple bacteria: paving the way for protein production on fermented wastewater

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Declaration of interest

None.

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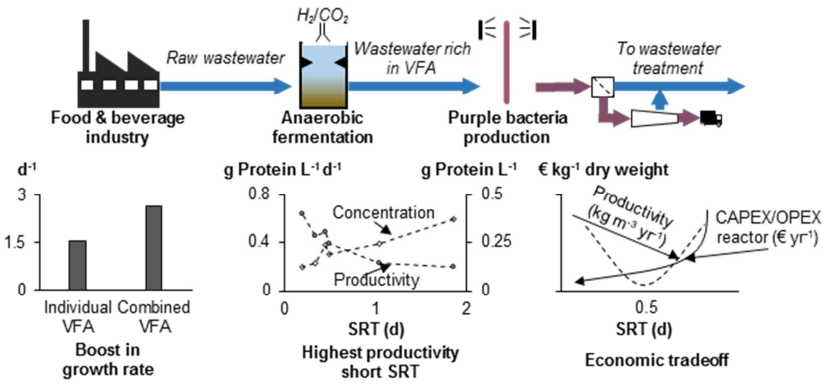
Abstract

Nutrient losses in our food chain severely surpass our planetary boundaries. Resource recovery can contribute to mitigation, for instance through converting wastewater resources to microbial protein for animal feed. Wastewater typically holds a complex mixture of organics, posing a challenge to selectively produce heterotrophic biomass. Ensuring the product's quality could be achieved by anaerobic generation of volatile fatty acids (VFAs) followed by photoheterotrophic production of purple non-sulfur bacteria (PNSB) with infrared light. This study aimed to determine the most suitable PNSB culture for VFA conversion and map the effect of acetate, propionate, butyrate and a VFA mixture on growth and biomass yield. Six cultures were screened in batch: (i) *Rhodopseudomonas palustris*, (ii) *Rhodobacter sphaeroides*, (iii) *Rhodospirillum rubrum*, (iv) a 3-species synthetic community (i+ii+iii), (v) a community enriched on VFA holding *Rb. capsulatus*, and (vi) *Rb. capsulatus* (isolate 'v'). The VFA mixture elevated growth rates with a factor 1.3-2.5 compared to individual VFA. *Rb. capsulatus* showed the highest growth rates: 1.8-2.2 d^{-1} (enriched) and 2.3-3.8 d^{-1} (isolated). In a photobioreactor (PBR) inoculated with the *Rb. capsulatus* enrichment, decreasing sludge retention time (SRT) yielded lower biomass concentrations, yet increased productivities, reaching 1.7 g dry weight (DW) L^{-1} d^{-1}, the highest phototrophic rate reported thus far, and a growth rate of up to 5 d^{-1}. PNSB represented 26-57% of the community and the diversity index was low (3-7), with a dominance of *Rhodopseudomonas* at long SRT and *Rhodobacter* at short SRT. The biomass yield for all cultures, in batch and reactor cultivation, approached 1 g $COD_{Biomass}$ g^{-1} $COD_{Removed}$. An economic estimation for a two-stage approach on brewery wastewater (load 2427 kg COD d^{-1}) showed that 0.5 d SRT allowed for the lowest production cost (€10 kg^{-1} DW; equal shares for capex and opex). The findings strengthen the potential for a novel two-stage approach for resource recovery from industrial wastewater, enabling high-rate PNSB production.

Keywords: short-chain fatty acids, single-cell protein, carboxylate platform, phototrophic purple bacteria, carbon capture

Graphical abstract



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1 Introduction

The conventional fertilizer–feed–food–fork chain is a cascade of inefficiencies (Steffen et al., 2015). For every 100 units nitrogen or phosphorus fertilizer applied to the land, only 14 units are consumed by citizens (Coppens et al., 2016). Upgrading resources from wastewater to microbial biomass could improve the efficiency of the food chain, with this protein-rich product used as ingredient in animal feed preparations. In this domain, affordability implies the need for non-axenic cultivation of microbial communities.

Wastewater contains organics, and heterotrophic microorganisms are therefore required to upgrade these streams to protein. However, wastewater typically holds a variable mixture of organic molecules of diverse complexity such as polysaccharides, proteins, fatty-acids, *N*-acetylamino sugars, DNA and polyphenolic structures (Dignac et al., 2000), posing a challenge to selectively and reproducibly produce heterotrophic biomass. Moreover, from a market perspective, resource recovery will be most attractive if microbial protein can be produced controllable with a constant quality (Verstraete et al., 2016). Therefore, a two-stage approach was conceptually proposed, anaerobically converting organics to volatile fatty acids (VFAs) followed by microbial protein production (Alloul et al., 2018).

Anaerobic fermentation is defined as the conversion of more complex organics to a mixture of VFA, rich in acetate, propionate and butyrate (Agler et al., 2011, Cagnetta et al., 2016). This conversion entails hydrolysis of particulate matter, followed by the production of acids (acidogenesis and acetogenesis) and CO_2 and H_2. Methanogenesis, i.e. CH_4 formation, is avoided (Agler et al., 2011). This microbiological process allows to ‘normalize’ the organic variability over time (Ahn et al., 2001, Banerjee et al., 1999) and the produced VFA with the corresponding low pH might act as a means to reduce contamination of the influent. The toxicity of VFA for microorganisms is well described in literature (Sun and O'Riordan 2013)

and anaerobic digestion leads to a die-off of pathogens (Wagner et al., 2008). Similar results might therefore be expected for anaerobic fermentation.

Two metabolic types of heterotrophic bacteria are of particular interest for protein production, namely aerobic heterotrophic bacteria and photoheterotropic bacteria (Verstraete et al., 2016). Aerobic heterotrophs have been explored for microbial protein production from the early 1950s (Vriens et al., 1989) and are pioneering in both research and pilot implementation (Lee et al., 2015, Matassa et al., 2016a). They are characterized by high growth rates (2-6 d^{-1}) and yields of 0.44-0.55 g $C_{Biomass}$ g^{-1} $C_{Removed}$ (Tchobanoglous et al., 2003). Photoheterotropically grown purple non-sulfur bacteria (PNSB) are also promising for microbial protein. They perform anoxygenic photosynthesis and use organics as electron donor and carbon source. Unlike algae, they are not able to use water as electron donor, but require molecules with lower redox potential than water such as VFAs (Blankenship et al., 1995). They grow at rates between 1.51-1.69 d^{-1} for mixed cultures (Hülsen et al., 2014, Kaewsuk et al., 2010) and between 0.96-7.10 d^{-1} for pure cultures (Noparatnaraporn et al., 1987, Ponsano et al., 2008). In addition, substrate-to-biomass organic C conversion yield is around one (0.8-1.2 g $C_{biomass}$ g^{-1} C_{fed} (Nakajima et al., 1997, van Niel 1944). Compared to aerobic heterotrophs, PNSB offer an additional steering tool to manage the microbial selectivity due to their unique ability to grow under anaerobic conditions on infrared (IR) light. In the case of sewage treatment for instance, with IR irradiation, a PNSB abundance of up to 90% was achieved (Hülsen et al., 2016a, Hülsen et al., 2016b).

To date, there are a number of research articles focusing on one-stage, direct, PNSB production on different types of industrial wastewater and domestic wastewater with pure cultures and open mixed cultures (Hülsen et al., 2016b, Kornochalert et al., 2014, Liu et al., 2016, Loo et al., 2013, Lu et al., 2013, Ponsano et al., 2011, Wu et al., 2015). For a two-stage approach, with anaerobic fermentation preceding the photoheterotrophic reactor, only two

approaches are available, starting from solid streams, implementing the fermentation stage as a liquefaction stage. One study has investigated fecal matter and plant material (Clauwaert et al., 2017) and another settled/diluted kitchen waste (Wang et al., 2016). On industrial wastewater from food/beverage, with the organics mainly in solution, such two-stage approach has not yet been explored. Previous studies that have investigated PNSB in a VFA-based medium, have studied the VFA biomass yield relationship for *Rhodobacter sphaeroides* (Nakajima et al., 1997, van Niel 1944), the effect of a VFA mixture on polyhydroxyalkanoate accumulation with an enrichment community holding *Rhodopseudomonas* sp. (Fradinho et al., 2014) and the molecular mechanism of VFA assimilation for *Rhodospirillum rubrum* (De Meur et al., 2018). A broader screening of PNSB for VFA growth kinetics and microbial selectivity imposed by VFA during non-axenic cultivation has according to the authors' knowledge not been investigated. Therefore, the first goal of this research was culture selection by determining the fastest growing PNSB on VFA along with their protein content and biomass yield. Six different cultures were investigated of which three pure cultures, a 3-species synthetic community to study potential synergetic effects, a community enriched on a VFA mixture to obtain a PNSB community specialized in VFA conversion and the isolated species to verify the findings of the latter. Secondly, this research wanted to unravel whether individual VFA or a VFA mixture had an impact on growth kinetics since anaerobic fermented wastewater typically consists of a VFA mixture. Afterwards, the enrichment community, which had obtained a very good performance during the batch test, was used as inoculum in a photobioreactor (PBR). Here, it was the objective to investigate the effect of sludge retention time (SRT) on microbial community dynamics and protein productivity. The findings of the PBR were finally used as input for an economic based optimization of the SRT. Production cost for anaerobic fermentation of brewery wastewater, PNSB cultivation, harvesting and drying were estimated and operational strategies were evaluated.

2 Materials and methods

2.1 PSNB strains and communities

To screen for the best PNSB culture for growth on VFA, six cultures were pre-selected: (i) *Rhodopseudomonas palustris* LMG 18881, (ii) *Rhodobacter sphaeroides* LMG 2827 and (iii) *Rhodospirillum rubrum* S 1H along with (iv) a 3-species synthetic community (i+ii+iii), (v) an enrichment community and (vi) *Rb. capsulatus* isolated from the latter through dilution to extinction. The pure cultures were axenically pre-cultivated in a climate chamber (Snijders Scientific) with a pre-autoclaved VFA-based medium adapted from Imhoff (2006). The details are presented in Supplementary Information S1.

The enrichment community was obtained by incubating a 1/1/1 volatile suspended solids ratio (0.1 g VSS L^{-1} each) of activated sludge from a sewage treatment plant, activated sludge from a dairy wastewater treatment plant and sediment from a local pond with a VFA mixture and IR light to acquire a PNSB culture specialized in VFA conversion. After twenty-four hours of incubation carotenoid (320 and 380 nm) and bacteriochlorophyll peaks (590, 800 and 880 nm) appeared in the spectrum. The centrifuged biomass was then characterized by high-throughput 16S rRNA sequencing (Illumina MiSeq; V4 region).The details are presented in Supplementary Information S2.

2.2 Batch experiments

The growth of the six abovementioned cultures (four strains and two communities) was examined in a 500 mL Erlenmeyer with a working volume of 400 mL through batch incubation. The experiments for the pure cultures and the 3-species synthetic community were performed in the climate chamber (conditions ibid. Supplementary Information S1). The experiment with the enrichment community was executed on a bench with IR transmission filters (conditions ibid. Supplementary Information S2). Three individual VFA were tested in triplicate (equal amount of carbon) along with a VFA mixture for every species and

community: (i) 0.73 g acetic acid L^{-1}, (ii) 0.54 g propionic acid L^{-1}, (iii) 0.60 g butyric acid L^{-1} and (iv) combined VFAs (1/1/1 ratio on carbon mass basis: 0.24 g acetic acid L^{-1}, 0.20 g propionic acid L^{-1} and 0.18 g butyric acid L^{-1}). The pH was adjusted to 7.0 prior to the experiment by the addition of 12 M NaOH. The flasks were then inoculated at an optical density of 0.1 (absorbance at 660 nm). The growth was monitored by measuring the absorbance at 660 nm. Samples were taken at the start (15 ml) and the end of the test (100 mL).

2.3 Photobioreactor experiment

A non-axenic semi-continuous PBR was operated to study the effect of SRT on microbial community dynamics and protein productivity. The PBR was a vertical tubular vessel with a working volume of 2.75 L and an external diameter of 12 cm. It was operated for 51 days at a temperature of 30 ± 2 °C and illuminated with two halogen lamps at a light intensity of 30 W m^{-2} covered with IR transmission filters. Stirring was done with a magnetic stirrer at 720 rpm (Fisher Scientific, USA). The reactor was inoculated with biomass from the enrichment community. The headspace of the reactor was flushed with nitrogen gas and the gas outlet was connected to a nitrogen gas expansion balloon in order to cope with underpressure and overpressure during withdrawal and fill. Influent and effluent tubing was covered with aluminum foil to minimize phototrophic growth.

A growth experiment of forty-eight hours with a VFA mixture (1/1/1 ratio on carbon mass basis: 0.73 g acetic acid L^{-1}, 0.60 g propionic acid L^{-1} and 0.54 g butyric acid L^{-1}) was performed prior to reactor operation to determine the maximal possible biomass concentration before light limitations occurred (Supplementary Information S3). The maximal biomass concentration was converted to a VFA concentration at which growth would not be limited by carbon (i.e. 1 g chemical oxygen demand; COD L^{-1} by assuming a yield of 1 g $COD_{Biomass}$ g^{-1} COD_{VFA}). The COD of the VFA mixture described in subsection 2.2 was adapted to 1 g COD

L^{-1} and used as influent for the reactor. The pH of the PBR was not controlled, yet the influent pH was lowered with 12 M HCl in order to have a final pH of 7.0 in the effluent (pH rises due to VFA consumption).

The reactor was operated at six different SRT by altering the volumetric flow rate (L d^{-1}). An overview of reactor conditions during operation is presented in Supplementary Information S4. The volume exchange ratio was initially set at 13% and later on altered to 25%. One up to four (four at short SRT) samples were taken daily to monitor the optical density (660nm), pH, temperature and electrical conductivity. Steady-state conditions were defined as a maximal change of 10% in optical density (proxy for biomass concentration), pH, temperature and electrical conductivity for three successive SRT. Samples for further analyses were taken three sequential SRT after steady-state conditions were reached.

2.4 Analytical procedures

The COD was measured using photometric test kits (Merck, Germany). The biomass yield was determined by measuring removed COD (i.e. soluble COD_{start} minus soluble COD_{end}) and biomass COD (i.e. total COD minus soluble COD). Protein was determined according to an adapted Lowry procedure (Markwell et al., 1978). VFA were analyzed by High Performance Liquid Chromatography (Agilent technologies 1200) coupled with a diode array detector (210 nm), a Bio-Rad Aminex® column (300 mm 7,8 mm) with Bio-Rad Micro-Guard Cation H Refill Cartridges and a column temperature of 40 °C. The samples were injected into 50 mM H_2SO_4 (Honeywell Fluka™) in deionised water (Arium® 611) with an injection volume of 20 µL and a sample flow rate of 0.6 mL min^{-1}. Total suspended solids (TSS) and VSS were determined according to standard methods (Greenberg et al., 1992). Handheld meters were used to measure pH (Hanna Instruments, USA) and electrical conductivity (Hanna Instruments, USA).

2.5 Molecular microbial and phylogenetic analyses

Molecular microbial analysis was used to characterize the most abundant PNSB in the enrichment, characterize the isolated species of the latter and follow-up richness, Shannon index, diversity and PNSB abundance during reactor operation. Phylogenetic relationships between the 16S rRNA gene sequence of the isolated species was done in order to classify the species.

A sample for microbial analysis was taken after four days of incubation. The most dominant species of the enrichment community was isolated using the dilution to extinction method. Biomass samples of the PBR were collected at three successive SRT when steady-state was reached. DNA was extracted using a PowerFecal® DNA isolation kit (QIAGEN, Germany) according to the manufacturer's instruction. The microbial community was analyzed using high-throughput 16S amplicon sequencing and a dual index strategy as described by Kozich et al., (2013). A detailed description can be found in Supplementary Information S5.

For the isolate, PCR products were sequenced using Sanger sequencing with both 8F and 1525R primers (Genetic Service Facility, Antwerp). Editing, complete overlapping and consensus construction was performed with the Geneious software. The consensus sequences were annotated with sequences in the EZBioCloud database (Chunlab Inc., Korea).

MEGA7 software was used to conduct phylogenetic analysis (Kumar et al., 2016). The phylogenetic tree was built using the Maximum Likelihood method based on the General Time Reversible model (Nei and Kumar 2000). The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were obtained automatically by applying the Maximum Parsimony method. In total, 1000 bootstrap replications were performed to test for branch robustness.

2.6 Statistical analyses

Multiple comparisons were performed using the parametric analysis of variance test and post-hoc pairwise comparisons using the Tukey's range test. Homogeneity of variances was verified by executing a Levene's test and normality of data residuals using the Shapiro–Wilk normality test. The non-parametric Kruskal-Wallis rank sum test and post-hoc pairwise comparisons using the Mann-Whitney U test (*p-values* were adjusted using the Benjamini-Hochberg correction) were performed when normality was rejected. A significance level of $p < 0.05$ was chosen. All analyses were performed in R using RStudio (RStudio®, USA) for Windows (R Core Team 2017).

2.7 Economic estimation

An economic evaluation was performed to validate the optimal SRT for PNSB production, according to a four-step methodology which is thoroughly described in Supplementary Information S6. The following parameters were taken into account such as construction, piping, PBR, circulation pump, ultrafiltration unit, centrifuge, spray dryer etc. An overview of all cost parameters is presented in Supplementary Information Table S1. After PNSB production, wastewater still need to be treated to reach effluent discharge limits. The wastewater treatment cost that are prevented due to PNSB production (remove part of COD) are taken into account in the model, yet valorization of biogas is not included. Process steps that are animal specific such as nucleic acid removal, endotoxin removal and pasteurization were not taken into account in the estimation. In addition, our process is an open systems and thus potentially prone to pathogens. Therefore, we opt for a multiple barrier principle as discussed by Alloul et al., (2018) which entails the following steps: i) inactivation of pathogens during anaerobic fermentation, ii) solid/liquid separation after anaerobic fermentation iii) selective culture conditions (PNSB on infrared light), iv) drying and pasteurization of biomass.

The primary goal of this model was to determine the best SRT to operate the PBR. It was not intended to determine an accurate production cost of PNSB for brewery wastewater on full-scale which than can be used for other research. This cost estimation ought to be seen as a decision making tool for R&D and the corresponding production scheme can be used as example for further research.

3 Results and discussion

3.1 Enrichment community

The results of the microbial community analysis of the PNSB enrichment can be found in Supplementary Information S5 and show an almost complete dominance of PNSB (85% relative abundance). The community was dominated by an amplicon sequence variant with a relative abundance of 80% which was classified as *Rhodobacter/Gemmobacter* (EZBioCloud database). Two other PNSB genera were also present although in low abundance (3% *Rhodopseudomonas*, 2% *Pararhodospirillum*). Of note, the short V4 region of the 16S rRNA gene probably does not allow to differentiate between some *Rhodobacter/Gemmobacter* species. To further characterize the most abundant PNSB and determine whether the amplicon sequence variant corresponds to *Rhodobacter* or *Gemmobacter*, a dilution to extinction method was applied to obtain an isolate which was subjected to Sanger sequencing of the full 16S rRNA gene (~1500 bp). Phylogenetic analysis of this sequence together with other closely related species showed that this isolate could be classified as a *Rhodobacter capsulatus* strain (Figure 1).

Due to dominance of *Rb. capsulatus* in the enrichment, we expected that growth performance of this strain would be above par. Growth rates for *Rb. capsulatus* between 4.9-5.4 d^{-1} are described in literature (Willison 1988), which are lower than values found for *Rb. sphaeroides* (7.4 d^{-1}) (Sangkharak and Prasertsan 2007). However, both results are for pure cultures

derived from mutant-strains. It is therefore difficult to draw conclusive statement about the growth performance of *Rb. capsulatus* on VFA. Therefore, in section 3.2 we will compare growth rates for several culture on individual and combined VFAs.

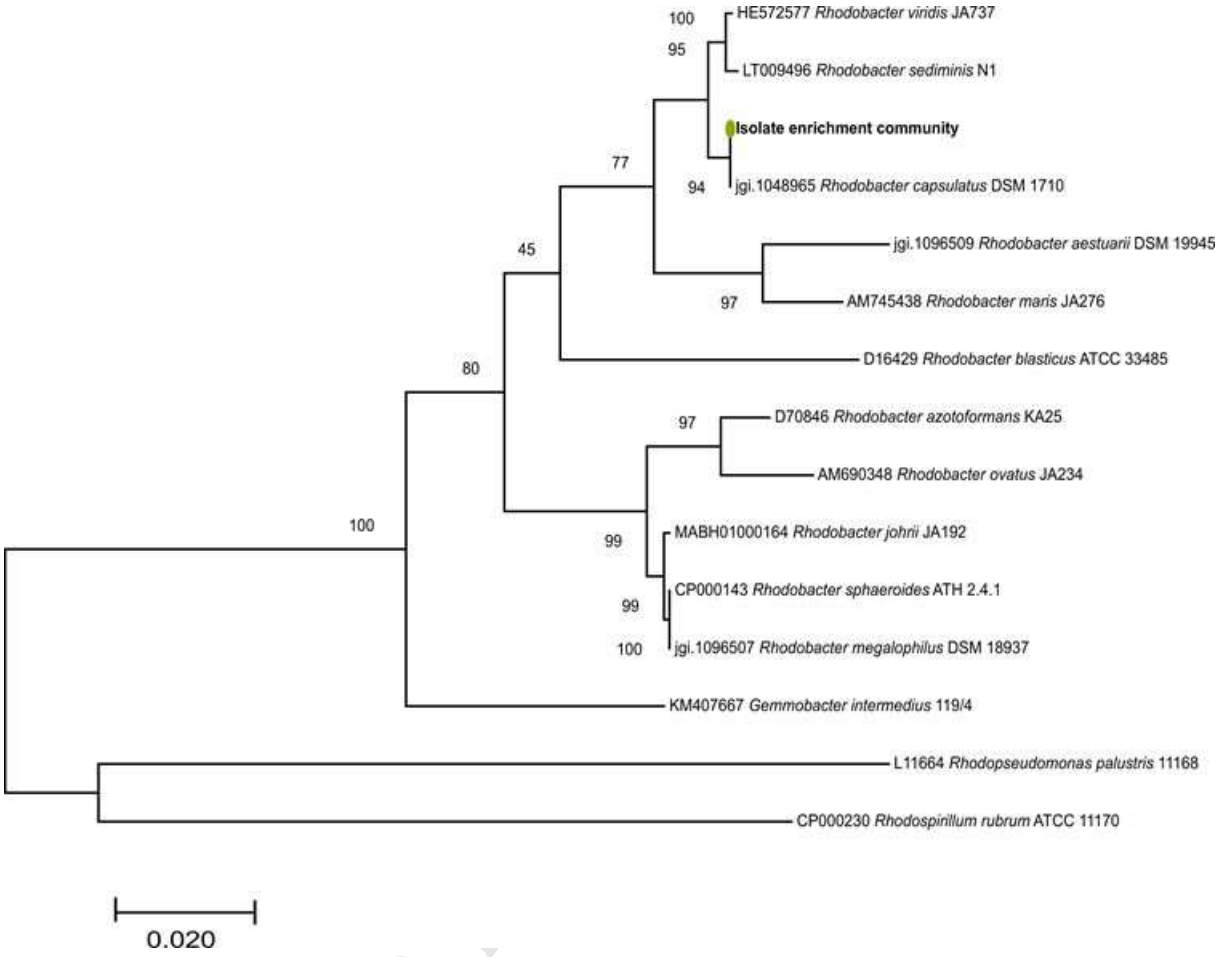

Figure 1 Phylogenetic relationship between the 16S rRNA gene sequence of an isolate of the enrichment, the three tested species, other species with 100, 99 and 98% similarity blast, and other *Rhodobacter capsulatus* strains (EZBioCloud database)

3.2 PNSB growth kinetics for individual and combined VFA

Batch experiments were performed to explore the effect of individual and combined VFAs on the biomass yield and growth rate, and screen within the six cultures for the most productive, i.e. fastest growing microorganism on VFA.

Figure 2 presents the biomass yield which was overall between 0.9-1.1 g $COD_{Biomass}$ g^{-1} $COD_{Removed}$. There was no significant difference (p > 0.05) between individual VFA or

individual and combined VFAs or between different species and communities. PNSB have a biomass yield equal to 1 g $COD_{biomass}$ g^{-1} $COD_{removed}$ and even higher due to photo-assimilation of a highly reduced electron donor (i.e. propionate and butyrate) along with CO_2 that serves as electron sink to compensate for excess reducing power (Blankenship et al., 1995). Nakajima et al., (1997) and van Niel (1944) have also observed a biomass yield of respectively 0.8 and between 0.8-0.9 g $COD_{Biomass}$ g^{-1} $COD_{Removed}$, yet these researchers only studied *Rb. sphaeroides*. The near-perfect substrate-to-biomass conversion is a critical advantage of PNSB compared to aerobic heterotrophs as roughly a double production potential is possible with a given amount of COD in the wastewater (yield 0.57 g $COD_{biomass}$ g^{-1} $COD_{removed}$; Tchobanoglous et al., 2003).

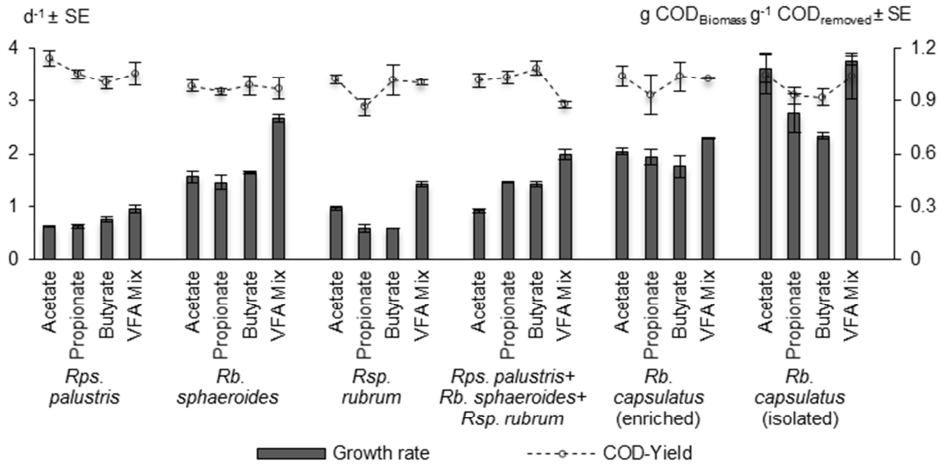

Figure 2 Overview of growth rate (left y-axis) and yield (right y-axis) for six cultures for individual and combined volatile fatty acids (VFAs). Error bars show standard error; COD: chemical oxygen demand.

For the growth rate, a change in individual VFA had no effect, as observed for all species and communities (Figure 2). The most remarkable observation during the batch test is the boost in growth rate when PNSB are fed with combined VFAs. This enhanced growth rate effect was noticeable for *Rps. palustris*, *Rb. sphaeroides*, *Rsp. rubrum* and the 3-species synthetic

community. The boost in growth rate was between 1.3-2.5 times higher compared too individual VFA, most pronounced for *Rsp. rubrum*. The absolute value for the enriched *Rb. capsulatus* and the isolated *Rb. capsulatus* was higher for combined VFAs, yet there was no significant difference between acetate and the VFA mixture ($p > 0.05$). The study of Nakajima et al., (1997) did not observe a growth rate enhancement effect for individual VFA (acetate, propionate and butyrate) compared to three different combined VFA ratios (1/1/1; 2/1/1 and 12/2/1 g carbon for respectively acetate, propionate and butyrate). This is probably due to the lack of sampling points during the exponential phase resulting in flattening down of the exponential correlation. Moreover, only *Rb. sphaeroides* was tested without any replicates, which makes it difficult to draw general conclusions. More recently, the study of Fradinho et al., (2014) has observed higher growth rates (consumption rate multiplied by biomass yield) for combined VFAs (1.53 d^{-1}) compared to acetate (1.22 d^{-1}) in line with our results. They studied the effect of a VFA mixture (4/1/1 g carbon) on polyhydroxyalkanoate accumulation with an enrichment community holding *Rhodopseudomonas* sp. The effect of combined VFAs is probably due to the presence of acetate as co-substrate. Without acetate the consumption of propionate and butyrate is limited by an internal acetyl-CoA deficit (Fradinho et al., 2014). Overall, our study is the first broader screening of PNSB for growth on VFA. The results highlight that a VFA mixture, and therefore likely fermented wastewater, is a more suitable substrate to produce PNSB at high rate compared to individual VFA. However, the VFA ratio can change for a fermentate depending on the operational conditions (Lee et al., 2014). Future research should therefore further unravel the effects of different VFA carbon ratios.

The isolated *Rb. capsulatus* had the highest growth rates for all individual and combined VFAs. Therefore, this culture might have the best potential to grow on fermentate. Due to the context of the application, which is resource recovery on wastewater, we choose to proceed

with a non-axenic reactor and non-axenic culture, which is the enriched *Rb. capsulatus*. Growth rates of the isolated *Rb. capsulatus* were 1.3-1.8 times higher compared to the enriched *Rb. capsulatus*. A similar observation can be made for the 3-species synthetic community which was the mixture of *Rps. palustris, Rb. sphaeroides, Rsp. rubrum*. Growth rates of *Rb. sphaeroides* (best individual performance of three species) for acetate and the combined VFAs were respectively 1.4 and 1.7 times higher compared to the 3-species synthetic community. This compares to the results of the *Rb. capsulatus* isolate which also had higher growth rates compared to the enrichment community (mixed culture). PNSB species share the same resources, therefore a competitive interaction in communities could be present which might result in detrimental effects for all species (Little et al., 2008).

The results of this study are also relevant for regenerative life support systems such as the MELiSSA loop, currently using the PNSB *Rsp. rubrum* for VFA conversion (Clauwaert et al., 2017). Our results point towards *Rb. capsulatus* as a more suitable culture for intensified protein production. Indeed, *Rb. capsulatus* is 2.6 times faster compared to *Rsp. rubrum*, and will allow for a more compact and lightweight bioreactor, a key advantage for space missions. However, also other factors ought to be taken into account for culture selection such as the broader metabolic capabilities, production of metabolites and the ability to maintain genetic stability under more severe radiation (Clauwaert et al., 2017).

Figure 3 presents an overview of the biomass composition (protein, non-protein VSS and fixed suspended solids; FSS). No general trend could be observed in protein content between individual or individual and combined VFAs. The overall protein content of *Rsp. rubrum* was significant higher than *Rps. palustris*, *Rb. sphaeroides* and equal to the enriched *Rb. capsulatus* and isolated *Rb. capsulatus* and the 3-species synthetic community. Vrati (1984) observed protein-levels that were between 1 and 2 times higher than our results. Growth in a medium with carbohydrates and a higher nitrogen concentration might potentially affect the

protein content. Vrati (1984) used clarified cow dung slurry as medium which contained 4 g L^{-1} carbohydrates and 10 times more nitrogen than our medium. On the contrary, a VFA-based medium was used in our tests. The degree of biomass reduction or biomass COD:VSS ratio was on average 1.35 g COD g^{-1} VSS (Figure 3**)**, which is comparable with values of activated sludge (1.20-1.49 g COD g^{-1} VSS; Contreras et al., 2002).

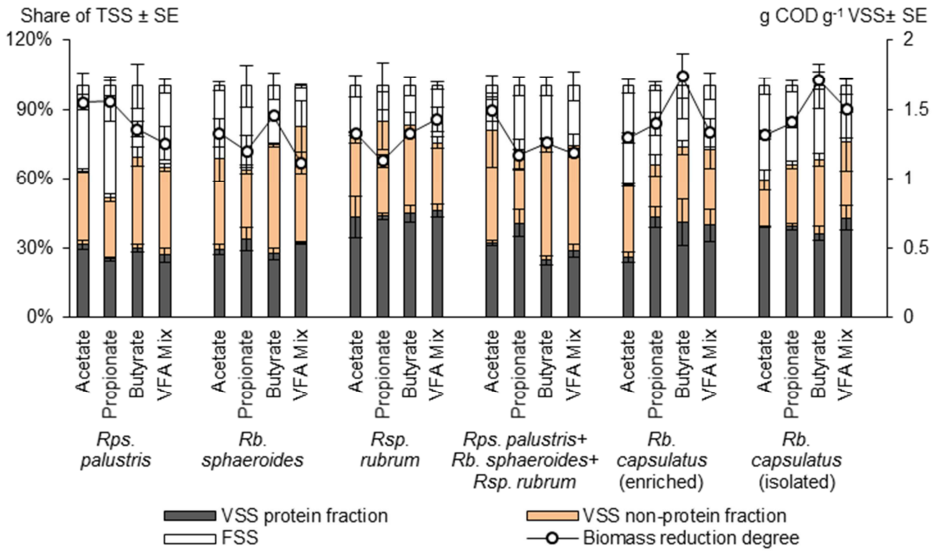

Figure 3 Biomass composition of all batch experiments (left y-axis), as breakdown of total suspended solids (TSS) into volatile suspended solids (VSS) protein fraction, VSS non-protein fraction, fixed suspended solids (FSS) and as biomass reduction degree (right y-axis). Error bars show standard errors.

3.3 Maximizing the protein productivity in a PBR on a VFA mixture

A non-axenic semi-continuous PBR was operated as chemostat, and the effect of SRT on microbial community dynamics and protein productivity (g protein L^{-1} d^{-1}) was studied. In the batch experiments, the *Rb. capsulatus* cultures had growth rates of 3.8 d^{-1} on combined VFAs. Therefore, it was expected that PNSB would be washed out at the corresponding dilution rate, representing a SRT below 0.3 d. However, a shorter SRT of even 0.19 d (4.6 h) was achieved and *Rhodobacter* was still abundantly present (**Figure** 4).

It was anticipated that implementation of high-rate conditions would impose an additional selective pressure on the microbial community favoring PNSB (cf. fast growth on VFA and IR light), leading to washout of slow growing non-PNSB microorganisms. The minimal attainable SRT of a species (i.e. $1/\mu_{max}$) is during washout higher than the actual SRT imposed on the reactor. Therefore, production of new cells will be lower than the amount that is washed out (Saikaly and Oerther 2004). This hypothesis was in line with the findings of Matassa et al., (2016b). They have operated a continuous bioreactor using hydrogen-oxidizing bacteria for protein production at a SRT of 10 h. The microbial community was almost completely dominated by hydrogen-oxidizing bacteria. However, washout effects might be more complex for mixed cultures than for pure cultures. For example, the SRT at which washout occurs can differ because the available COD for each species will be lowered by the presence of other species (Saikaly and Oerther 2004).

The results of 16S rRNA gene sequencing showed relatively high PNSB dominance during operation (relative abundance around 27-60%; Figure 4), albeit lower than the PNSB abundance in the inoculum enrichment community (85%, Supplementary Information Figure S3). Species richness was highest at a SRT of 2 d, decreased to 8 at a SRT of 0.5 d and finally increased again to 10-11 at SRT values between 0.2-0.3 d. No extra selective pressure favoring PNSB growth could be observed at shorter SRT based on the results of the diversity index. However, a selective microbial community (low diversity index) with relatively high PNSB dominance could be maintained over several SRT in the PBR.

Three PNSB genera could be detected namely *Rhodobacter*, *Rhodopseudomonas* and *Rubrivivax* of which the former two were most abundant. The SRT had an effect on relative abundance between different PNSB genera. The genus *Rhodopseudomonas* was most dominant at long SRT, while *Rhodobacter* at short SRT. A clear distinction between the enriched *Rb. capsulatus* community and the microbial community during reactor operation is

the strong presence of *Acinetobacter* (relative abundance around 25-56%). Members of this genus were also found in other studies investigating open mixed PNSB cultures with relative abundances of 17.3% (Wang et al., 2016), 0.05-0.6% (Hülsen et al., 2016a) and qualitative detected through DGGE followed by sequencing (Izu et al., 2001). These gram-negative bacteria are known for their ability to accumulate phosphate, as employed in the enhanced biological phosphorus removal process (Kim and Pagilla 2000). As phosphate accumulating organisms are typically stimulated with alteration in anaerobic and aerobic conditions. It is possible that oxygen entered the PBR during withdrawal of the effluent (higher headspace to volume ratio).

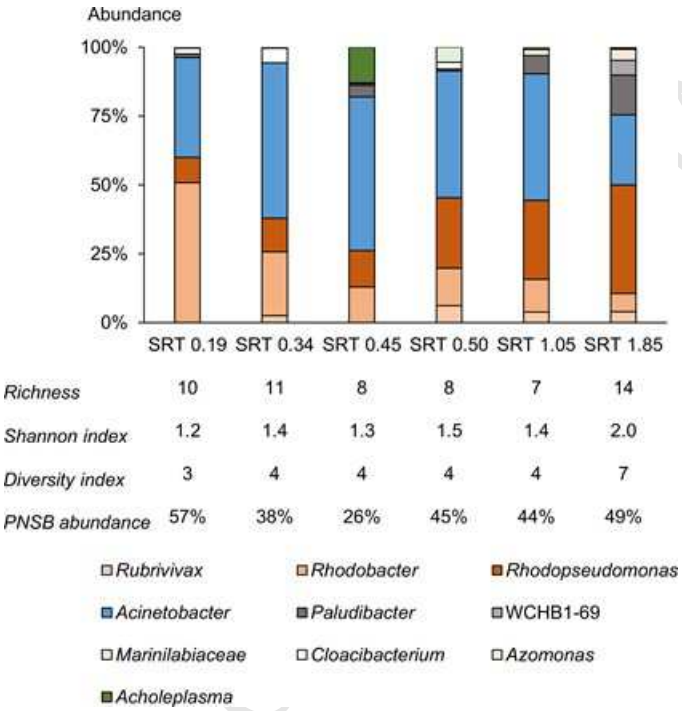

Figure 4 Effect of sludge retention time (SRT) on microbial community composition, richness, Shannon index, diversity index which is the exponential of the Shannon index and purple non-sulfur bacteria (PNSB) abundance during reactor operation. PNSB genera *Rhodobacter*, *Rhodopseudomonas* and *Rubrivivax* are all marked in orange colors.

Figure 5 presents an overview of the effect of SRT on protein concentration (g protein L^{-1}) and protein productivity (g protein L^{-1} d^{-1}, protein concentration multiplied by dilution rate). A general trend that can be observed is the decrease in steady-state protein concentration due to higher biomass washout imposed by shorter SRT, yet the protein productivity increased. The highest protein productivity was 0.64 g protein L^{-1} d^{-1} or expressed in dry weight 1.7 g TSS L^{-1} d^{-1} achieved at a SRT of 4.6 h. Carlozzi and Sacchi (2001) have also obtained high photoheterotrophic productivities (1.1-1.4 g TSS L^{-1} d^{-1}) in an outdoor PBR with *Rps. palustris* as inoculum on a VFA-based medium, yet results are still lower than our findings probably due to natural light-dark cycles. To conclude, the TSS productivity of 1.7 g TSS L^{-1} d^{-1} is according to the authors' knowledge the highest reported in a PBR for a PNSB enrichment community and the first research to realize high-rate PNSB production (short SRT).

An important parameter in designing a PBR on fermented wastewater is the biomass specific removal rate, which was 4.5 ± 1.4 g $COD_{Removed}$ g^{-1} $COD_{Biomass}$ d^{-1} (SRT 4.6 h). In literature values for acetate, propionate and butyrate of respectively 1.5-2.4, 2.0-1.3, 0.1-1.7 g $COD_{Removed}$ g^{-1} $COD_{Biomass}$ d^{-1} are reported (Fradinho et al., 2014, Puyol et al., 2017). These high specific removal rates make PNSB competitive to high-rate aerobic heterotrophic bacteria (1-6 g $COD_{Removed}$ g^{-1} $COD_{Biomass}$ d^{-1} (Meerburg et al., 2015). However, the attainable biomass concentrations are higher for aerobic heterotrophs (not limited by light), and therefore the volumetric COD removal rates will also be higher, compared to phototrophically grown PNSB.

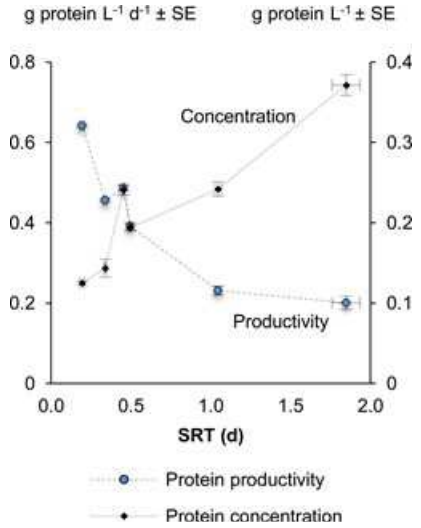

Figure 5 Effect of sludge retention time (SRT) on protein productivity (left y-axis) and protein concentration (right y-axis) for photoheterotrophic biomass production on VFA mixture (1/1/1 ratio on carbon mass basis: 0.73 g acetic acid L^{-1}, 0.60 g propionic acid L^{-1} and 0.54 g butyric acid L^{-1}) a semi-continuous photobioreactor (PBR). Error bars show standard error.

Figure 6 presents an overview of the VFA removal efficiency. All VFA are consumed at a SRT between 1-2 d, yet acetate and butyrate are washed out at a SRT of 0.5 d and lower. On the contrary, the removal efficiency for propionate remained almost unchanged at a SRT of around 0.5 d and was still 74 g $COD_{Removed}$ 100 g^{-1} COD_{Fed} at a SRT of 4.6 h. Hence, these results show that the microbial community had a preference for propionate over butyrate and acetate. De Meur et al., (2018) has studied binary VFA mixtures with *Rsp. rubrum* and observed a lag for butyrate uptake for the mixtures acetate/butyrate and propionate/butyrate, yet acetate and propionate were simultaneous assimilated. The lag in uptake is explained by De Meur et al., (2018) as a metabolic mismatch due to the fact that acetate and butyrate share acetyl-CoA and crotonyl-CoA as metabolic intermediates. It probably exists for microorganisms that are isocytrate lyase negative (ICL^-) and use other pathways for VFA assimilation (ethylmalonyl-CoA pathway) for example *Rsp. rubrum* and *Rb. sphaeroides*

(Alber et al., 2006, Kornberg and Lascelles 1960). It is difficult to extrapolate the findings of De Meur et al., (2018) to our results because it is not possible to verify which PNSB are ICL^+/ICL^- on a genus level and it is not known how non-PNSB species contribute to the specific VFA removal. More research is still required to unravel this at metabolic level for different PNSB. Recently Puyol et al., (2017) have published a phototrophic model for domestic wastewater treatment in anaerobic conditions. The effect of acetate/propionate on butyrate uptake is not included, yet our findings show that it can be crucial. New models should take these effects into account. These findings are also key when operating a PBR on fermented or on synthetic medium as acetate and butyrate washout can occur at short hydraulic retention time (HRT) or high loading rates.

Full VFA removal (100 g $COD_{Removed}$ 100 g^{-1} COD_{Fed}) at 1 and 2 d SRT showed that PNSB were actually underfed and thus probably higher volumetric productivities are achievable at these SRT. This was initially not observed because the soluble COD at a SRT of 1 and 2 d was around 230 mg COD L^{-1}, yet VFA analysis showed that the VFA concentration was zero. The excess in soluble COD could possibly be due to the production of extracellular enzymes (e.g. vitamin lipase, protease, amylase etc. ; Chumpol et al., 2017).

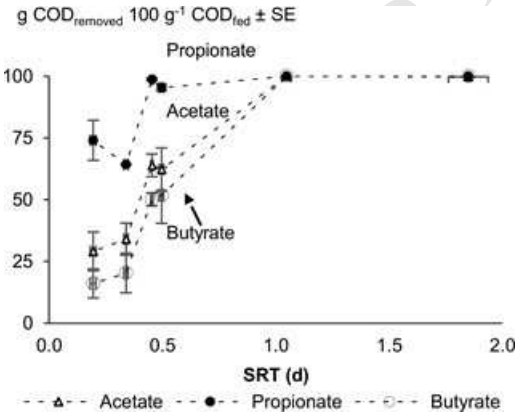

Figure 6 Volatile fatty acid (VFA) removal efficiency for acetate, propionate and butyrate as function of the sludge retention time (SRT). VFA mixture 1/1/1 ratio on carbon mass basis:

466 0.73 g acetic acid L^{-1}, 0.60 g propionic acid L^{-1} and 0.54 g butyric acid L^{-1}. Error bars show
467 standard errors (SE).

468 ### 3.4 Economic tradeoff to determine optimal SRT

469 The reactor cultivation showed an opposite influence of SRT on protein productivity and
470 harvestibility (biomass concentration; Figure 5). A cost estimation on brewery wastewater
471 corrected for normal costs of wastewater treatment (flow 1150 m^3 d^{-1} and COD-load 2427 kg
472 COD d^{-1}) was therefore performed to determine the optimum SRT for reactor operation,
473 including anaerobic fermentation, the PBR, downstream processing (harvesting and drying)
474 and final wastewater treatment after PNSB production to reach discharge limits. A process
475 scheme is presented in Supplementary Information S6 which depicts the production of PNSB
476 and the brewery wastewater treatment system after PNSB production.
477 The share of capital- and operational expenditure (CAPEX and OPEX) relative to total cost
478 was respectively between 51-59% and 41-49%. In general, both CAPEX and OPEX were
479 dominated by the cultivation stage, i.e. the PBR (Figure 7). With decreasing SRT, biomass
480 production increased from 107 to 342 tonnes m^{-3} yr^{-1}. Three production zones can be
481 recognized: (i) high productivity yet high expenses (SRT < 0.5 d), (ii) most economical
482 production (SRT of 0.5 d and cost of € 10 kg^{-1} dry weight; DW) and (iii) low productivity and
483 high expenses (SRT > 0.5 d).
484 The production cost of PNSB is comparable to microalgae (€ 5.96-12.6 kg^{-1} DW) (Acien et
485 al., 2012, Norsker et al., 2011), yet could be reduced if sunlight would be used. The CAPEX
486 for the artificial light system is not costly (0.5-1.5%), yet the energy for light is between 13-
487 28% of the OPEX. Sunlight PNSB production could theoretically lower the production cost to
488 € 9 kg^{-1} DW. PNSB production on sunlight is possible at high productivities as proven by
489 (Carlozzi et al., 2006). However for temperate climates it should be demonstrated that these
490 high productivities are still reached during colder and darker months.

PNSB production will reduce the COD concentration and therefore a portion of the wastewater treatment cost will be exempted. Overall, savings due to avoided treatment cost were between € 29,000-68,000 yr^{-1}, which accounts to 13-31% of the wastewater treatment costs without PNSB production. More savings are possible if productivities further increased because final wastewater treatment can then be simplified to aerobic treatment and no additional digester is needed. Decoupling SRT from HRT by a membrane bioreactor for example (Hülsen et al., 2016b) could increase the productivity due to higher volumetric removal rates imposed by a higher biomass concentration. For now, wastewater treatment after PNSB production with merely aerobic treatment was not possible because the effluent COD concentration remained too high (1.54 g COD L^{-1}) and nutrients too low (14 mg N L^{-1} and 0.3 mg P L^{-1}). If PNSB production was optimized, only aerobic treatment would be required. This would result in € 130,000 yr^{-1} extra savings for the treatment system (no nutrient addition taken in to account).

Overall, this cost assessment shows that a SRT of 0.5 d is most optimal for PNSB production, and production costs could potentially be optimized by sunlight.

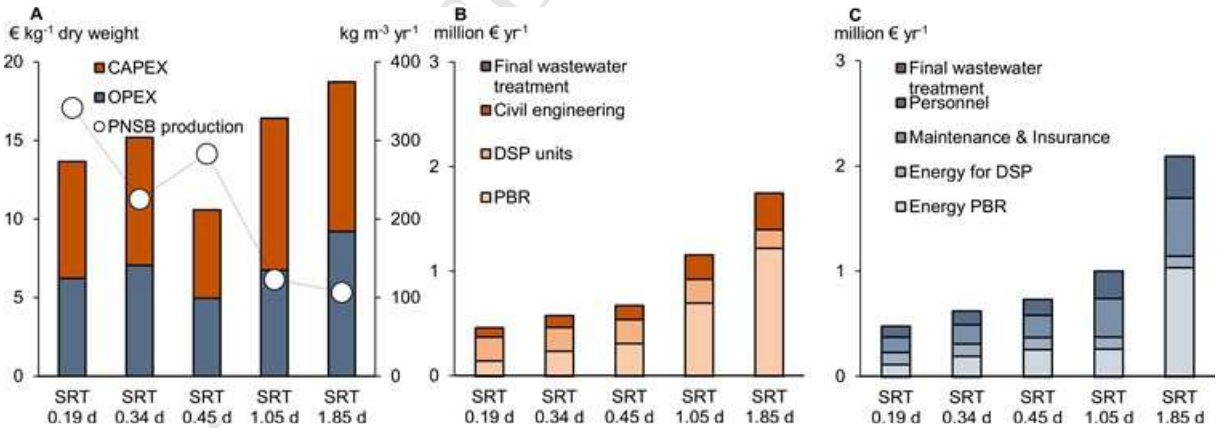

Figure 7 Economic tradeoff based on the production cost of photoheterotrophic biomass (left Y axis, A) as function of decrease in productivity (right Y axis, A) and increase in harvestability for longer sludge retention time (SRT). Distribution of capital expenditure

(CAPEX, B) and operational expenditure (OPEX, C). DSP: downstream processing, PBR: photobioreactor

4 Conclusions

(i) Combining the VFAs acetate, propionate and butyrate resulted in a boost in growth rate compared to the individual VFA, for all tested PNSB cultures. Anaerobic fermentate of wastewater consists of a VFA mixture, and might result in the same effect.

(ii) PBR experiments showed that propionate was the preferential VFA source when dosing combined VFAs. There is hence no use in overdosing acetate or butyrate in a synthetic medium or to steer fermentation to butyrate production. Acetate and butyrate washout can be problematic for a PBR at short HRT or at high loading-rates.

(iii) PNSB have a near-perfect substrate-to-biomass COD conversion when grown on VFA. This roughly doubles the production potential compared to aerobic heterotrophs.

(iv) This research is first to screen a broader range of PNSB for growth on VFA. From six PNSB cultures, the isolated and enriched *Rhodobacter capsulatus* obtained the highest growth rates.

(v) A biomass productivity of 1.7 g DW L^{-1} d^{-1} was achieved in the PBR, the highest rate reported for a phototrophic PNSB enrichment community with a focus on production.

(vi) Biomass-specific removal rates of 4.5 ± 1.4 g $COD_{Removed}$ g^{-1} $COD_{Biomass}$ d^{-1} were observed in the PBR. PNSB are therefore competitive to aerobic heterotrophic bacteria (alternative source of microbial protein) on a biomass-specific basis, yet cannot compete on a volumetric basis.

(vii) PNSB are able to cope with very short SRT. Their relative abundance was between 26-60% and the community diversity index was low (3-7). VFA and IR therefore seem suitable tools to enhance selectivity in a non-axenic PBR.

(viii) *Rb. capsulatus* might be a more suitable culture than *Rsp. rubrum* for regenerative life support systems for space with a focus on compact reactors.

(ix) The reactor cultivation showed an opposite influence of SRT (0.19-1.85 d) on protein productivity and harvestibility. An economic estimation for PNSB production enabled to the derivation of 0.5 days SRT as the most cost-efficient for biomass production, at a cost of € 10 kg^{-1} DW.

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ACCEPTED MANUSCRIPT

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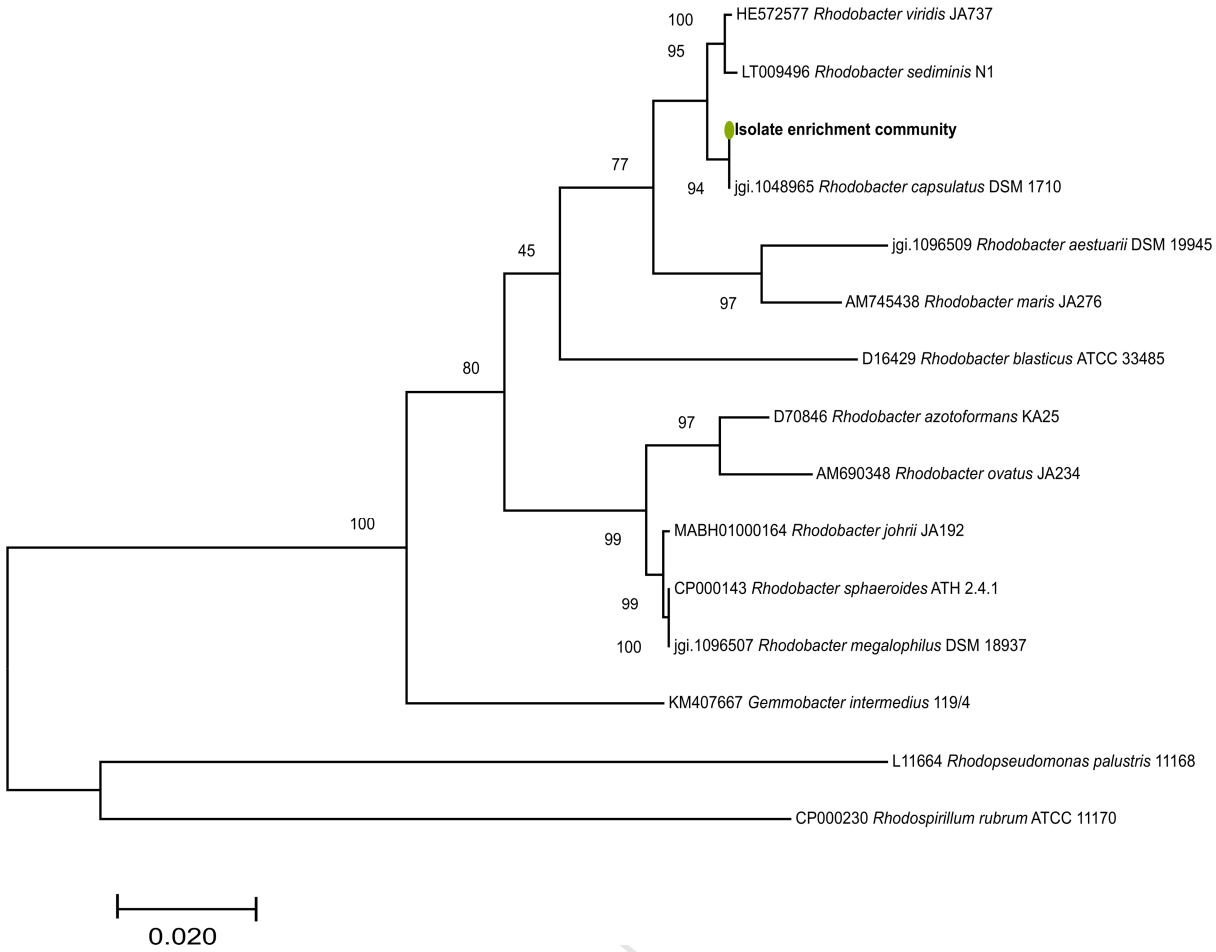

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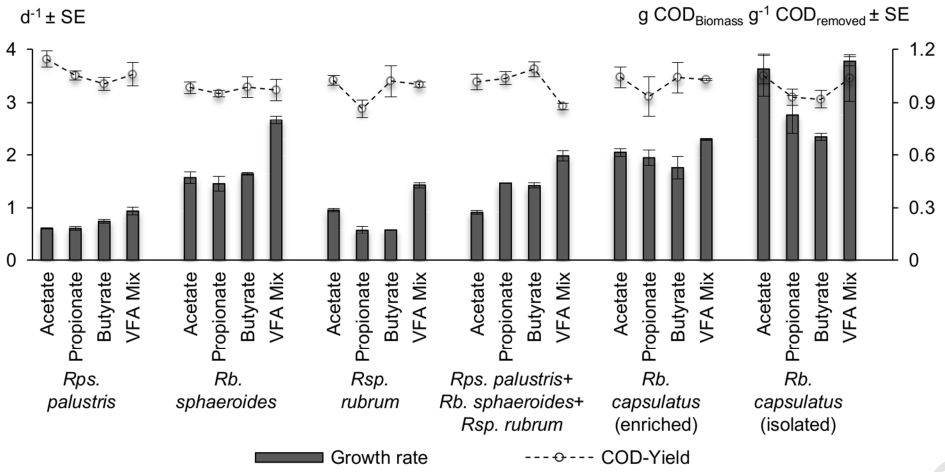

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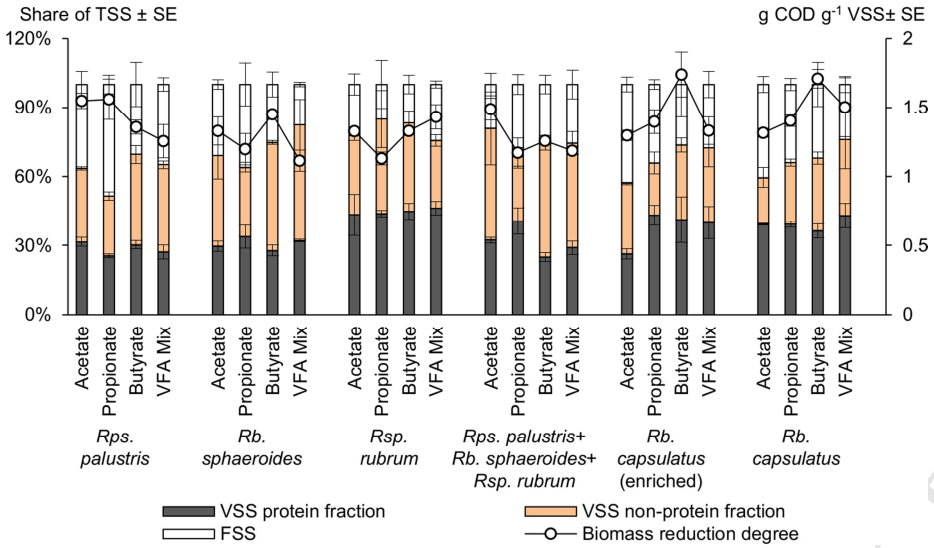

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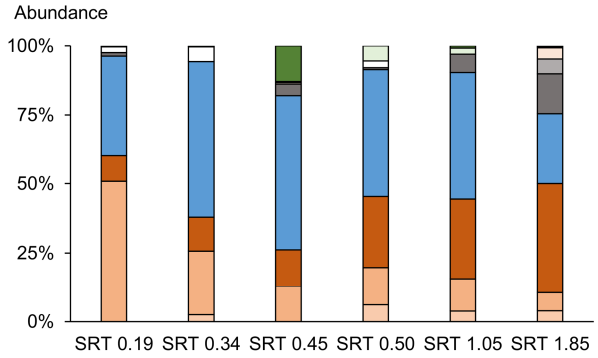

	SRT 0.19	SRT 0.34	SRT 0.45	SRT 0.50	SRT 1.05	SRT 1.85
Richness	10	11	8	8	7	14
Shannon index	1.2	1.4	1.3	1.5	1.4	2.0
Diversity index	3	4	4	4	4	7
PNSB abundance	57%	38%	26%	45%	44%	49%

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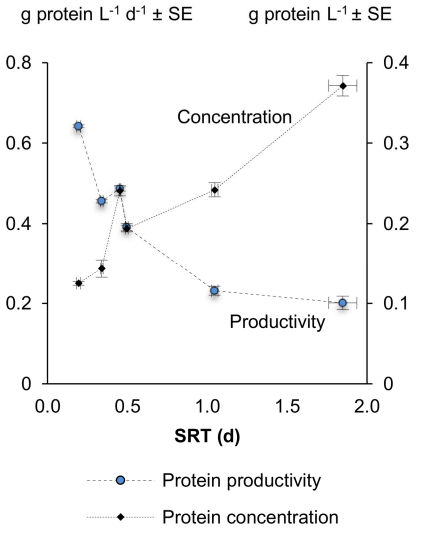

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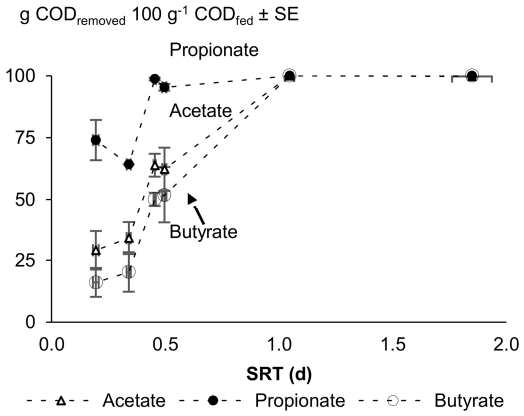

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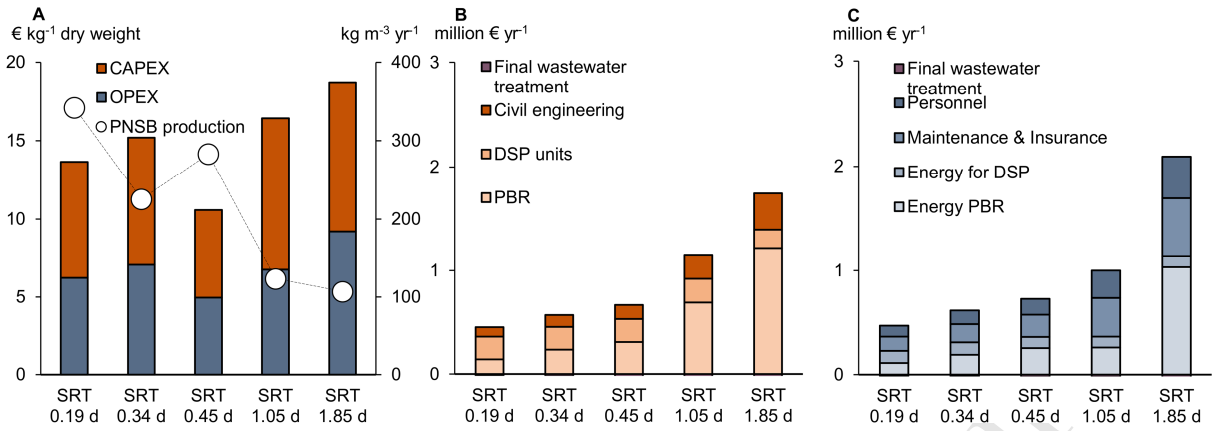

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Highlights

- Mixed rather than individual VFA boost growth of purple non-sulfur bacteria (PNSB)
- From six PNSB cultures, *Rhodobacter capsulatus* obtained the highest growth rates
- Highest volumetric productivity (1.7 g dry weight L^{-1} d^{-1}) reported for PNSB
- PNSB have a 1-to-1 or higher substrate-to-biomass conversion when grown on VFA
- PNSB most cost effective at sludge age of 0.5d based on model of brewery wastewater

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Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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