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Purple phototrophic bacteria for resource recovery: challenges and opportunities

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Abstract

Sustainable development is driving a rapid focus shift in the wastewater and organic waste treatment sectors, from a "removal and disposal" approach towards the recovery and reuse of water, energy and materials (*e.g.* carbon or nutrients). Purple phototrophic bacteria (PPB) are receiving increasing attention due to their capability of growing photoheterotrophically under anaerobic conditions. Using light as energy source, PPB can simultaneously assimilate carbon and nutrients at high efficiencies (with biomass yields close to unity (1 g $COD_{biomass}$·g $COD_{removed}^{-1}$)), facilitating the maximum recovery of these resources as different value-added products. The effective use of infrared light enables selective PPB enrichment in non-sterile conditions, without competition with other phototrophs such as microalgae if ultraviolet-visible wavelengths are filtered. This review reunites results systematically gathered from over 177 scientific articles, aiming at producing generalized conclusions. The most critical aspects of PPB-based production and valorisation processes are addressed, including: (i) the identification of the main challenges and potentials of different growth strategies, (ii) a critical analysis of the production of value-added compounds, (iii) a comparison of the different value-added products, (iv) insights into the general challenges and opportunities and (v) recommendations for future research and development towards practical implementation. To date, most of the work has not been executed under real-life conditions, relevant for full-scale application. With the savings in wastewater discharge due to removal of organics, nitrogen

and phosphorus as an important economic driver, priorities must go to using PPB-enriched cultures and real waste matrices. The costs associated with artificial illumination, followed by centrifugal harvesting/dewatering and drying, are estimated to be 1.9, 0.3-2.2 and 0.1-0.3 $\cdot kg_{dry\ biomass}^{-1}$. At present, these costs are likely to exceed revenues. Future research efforts must be carried out outdoors, using sunlight as energy source. The growth of bulk biomass on relatively clean wastewater streams (*e.g.* from food processing) and its utilization as a protein-rich feed (*e.g.* to replace fishmeal, 1.5-2.0 $\cdot kg^{-1}$) appears as a promising valorisation route.

Graphical abstract
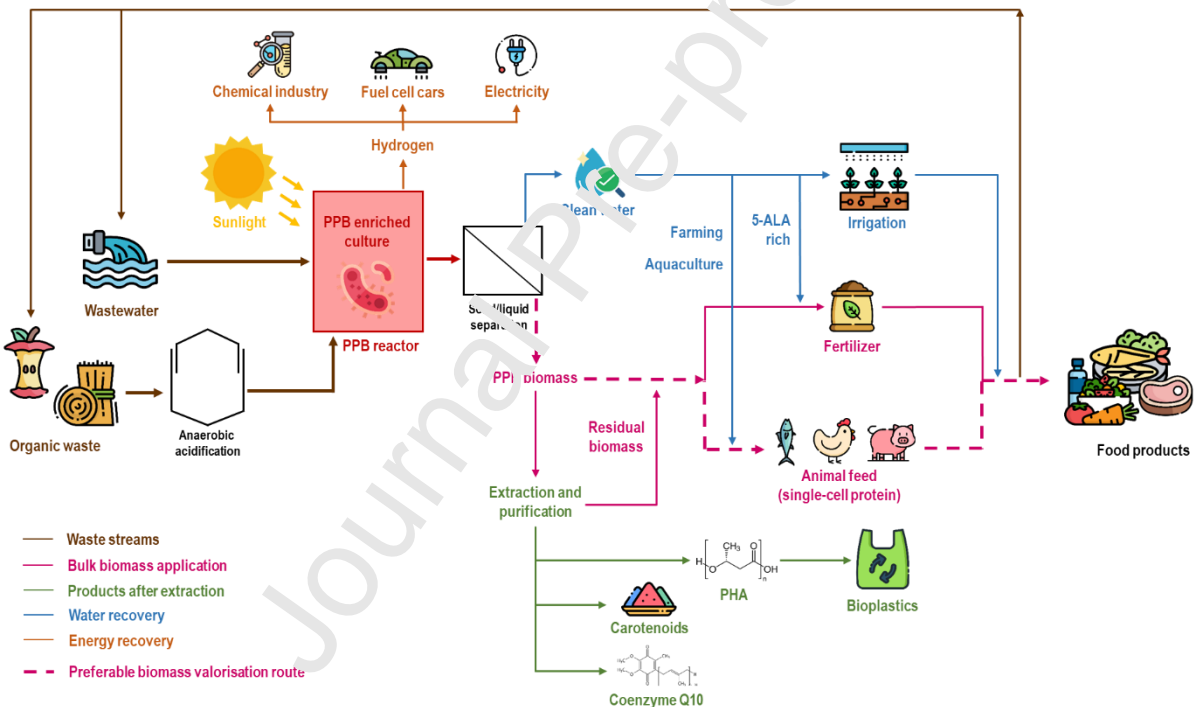
Keywords

Carotenoids; hydrogen; nutrient recovery; polyhydroxyalkanoates; purple non-sulfur bacteria; purple sulfur bacteria; single-cell protein; waste; wastewater

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1. Introduction

The need for sustainable development and efficient resource utilisation is driving a shift in the organic waste and wastewater treatment technologies, from environmental and public health protection at the cost of resource destruction, towards retaining these resources within the industrial ecosystem. For more than a century, conventional technologies have effectively removed organics, nutrients and pathogens from waste streams. Nowadays, the establishment of a circular and bio-based economy focused on resource recovery and reuse is a key social and technological challenge (Batstone et al., 2015). As part of this transition, the recovery and production of water, energy and materials based on organics and/or nutrients (*i.e.* nitrogen and phosphorus) from different waste streams, is becoming a major development driver. This must be done without compromising environmental protection and public health. Biological concentration via assimilative and/or accumulative partitioning has been proposed as an option to minimise the so-called dissipative removal of carbon via CO_2 and of reactive nitrogen as inert N_2, together with the removal of phosphorus as metal-bound precipitate (Batstone et al., 2015). These are critical and precious resources, and it is crucial to recover/upgrade them for re-use. In this context, photoheterotrophic mediators are particularly interesting, as they use energy from light to assimilate carbon and nutrients from waste streams at high efficiencies, enabling their recovery in the form of microbial biomass (Winkler and Straka, 2019) while avoiding carbon and nutrient dissipation, typical of chemoheterotrophic catabolism.

Microalgae, as oxygenic photosynthetic mediators, have been studied for several decades in photobioreactors (PBRs). While microalgae effectively assimilate N and P, the vast majority grow preferably photoautotrophically, using CO_2 as carbon source instead of organic compounds (Walker et al., 2005). Organic-rich substrates such as wastewaters, result in the development of mixed algae-bacteria cultures, with algae growth limited by light attenuation

at increased cell densities. For this reason, microalgae are predominantly applied for tertiary treatment (polishing step for further nutrient removal) (Posadas et al., 2015). Algal-bacterial consortia, such as ALBAZOD (algae, bacteria, zooplankton and detritus mixture) based systems have also been applied for secondary treatment, obtaining promising results (Robles et al., 2020b, 2020a). Nevertheless, challenges such as system instability and expensive biomass harvesting are still to be addressed (Hülsen et al., 2018b; Robles et al., 2020b). Despite the arsenal of value-added products that microalgae can generate (including cosmetics, carotenoids, single-cell protein (SCP), fertilizers, and biofuels (Lü et al., 2011; Melis, 2012)), the global microalgae production barely exceeds 10,000 $t_{dry\ biomass} \cdot year^{-1}$. Growing markets such as algal fertilizers (containing inherently biostimulants and biofungicides) have the potential to increase this number in the future. In addition, the vast majority of microalgae currently produced is not grown on recovered resources (*e.g.* waste streams) but on clean feedstocks, being dedicated to niche markets with very high value products (*e.g. Spirulina* for human consumption or astaxanthin as feed supplement) (de la Jara et al., 2018; Luque, 2010; Shah et al., 2016; Williams and Laurens, 2010). Consequently, microalgal bulk products, including protein or biofuels, have not reached mainstream applications. The main reason for this is the high production cost, exceeding 3.0-4.0 $\$ \cdot kg_{dry\ biomass}^{-1}$ when produced in open ponds, tubular or flat plate bioreactors (with drying being a major cost) (Acién et al., 2017; Norsker et al., 2011; Ruiz et al., 2016) or 2.0 $\$ \cdot kg_{biofuel}^{-1}$ produced from microalgae (Chen et al., 2018; Shirvani et al., 2011; Sun et al., 2019). To decrease these costs, the latest microalgal research has been directed towards (i) the utilization of waste streams as inexpensive or free-of-cost source of water, carbon and nutrients, (ii) cultivation in open-ponds and (iii) natural light as energy source (Goh et al., 2019; Kadir et al., 2018; Zhou et al., 2013). The costs calculations and contributions (capital and operational) have been detailed in several life cycle analyses and reviews (Acién et al., 2017, 2012; Acién

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Fernández et al., 2019; Beal et al., 2015; Norsker et al., 2012, 2011; Sun et al., 2019). This will be further analysed in relevant sections in this review.

The application of purple phototrophic bacteria (PPB) for resource recovery from waste streams is gaining increasing attention, specifically in mixed culture photoheterotrophic growth mode (Cao et al., 2020; Delamare-Deboutteville et al., 2019; Hülsen et al., 2018a, 2018b, 2016b). PPB are a diverse group of anoxygenic, phototrophic, facultative anaerobes. They are widely spread throughout the phylogenetic tree of bacteria, with many subdivisions, particularly within the Proteobacteria (Imhoff and Bias-Imhoff, 1995). PPB consist of purple sulfur bacteria (PSB) and purple non-sulfur bacteria (PNSB), which often coexist in the same environment (Madigan and Jung, 2009). PSB are predominantly photoautotrophic, using reduced sulfur compounds as electron donor to reduce inorganic carbon. PSB have limited photoheterotrophic and dark metabolic capabilities and most of them require sulfur for their growth (Madigan and Jung, 2009). In contrast, PNSB are broadly capable photoheterotrophs, with photoautotrophic capabilities in addition to diverse capacities for (aerobic/anaerobic) dark chemotrophy (Madigan and Jung, 2009). Other differences between PSB and PNSB include: (i) higher PSB tolerance to reduced sulfur species, (ii) the PSB capability to store S^0 intracellularly, and (iii) nitrogen fixation capabilities of PNSB (Madigan and Jung, 2009). Some PNSB representatives (*e.g. Rhodobacter sphaeroides*, *Rhodopseudomonas palustris*, and *Rhodospirillum rubrum*) have been studied for well over a century (Englemann, 1883), serving as model organisms to investigate the fundamentals of bio-photo energy generation and carbon and nitrogen fixation (Geyer and Helms, 2006).

The diverse range of potential PPB applications arises from the exploitation of their metabolic versatility, simplified in Figure 1 (the bold numbers in brackets given in this section refer to Figure 1A). PPB grow under anaerobic conditions, by **(1)** photoautotrophy, using light for anabolism and CO_2 as carbon source, with a range of inorganic electron donors, **(2)**

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photoheterotrophy, using light as energy source and organic carbon as carbon source, and **(3)** fermentation, without light and using organics as energy and carbon source. PPB are able to utilize a range of electron donors (such as: **(1)** autotrophic - H_2, H_2S, S_0, $S_2O_3^{2-}$, Fe^{2+}, NO_2^-, CO (Ehrenreich and Widdel, 1994; Griffin et al., 2007; Koku et al., 2002; Najafpour and Younesi, 2007), **(2)** heterotrophic, organic acids - acetate, propionate, butyrate, malate, succinate, lactate, dimethylsulfide, *etc.* (Kim et al., 2004; Lu et al., 2019b) and **(3)** heterotrophic - glucose, sucrose, lactose, ethanol, *etc.* (Ehrenreich and Widdel, 1994; Keskin and Hallenbeck, 2012; Madigan and Jung, 2009). In addition, they can **(4)** grow aerobically through respiration and fix dinitrogen gas via nitrogenase. Some (*e.g. Rhodobacter sphaeroides* or *Rhodopseudomonas palustris*) have also been reported to perform nitrification, denitrification (growing heterotrophically and using nitrate as electron acceptor) and to grow lithoautotrophycally (*e.g.* via halophilic S^{2-} oxidation) (Kim et al., 1999; Nagadomi et al., 2000; Puyol et al., 2020). As shown in Figure 1B, the environmental conditions (*e.g.* electron acceptor and electron donor present and presence/absence of light or oxygen) determine the predominant metabolic growth mode. In the presence of light and absence of oxygen, anoxygenic phototrophic growth **(1, 2)** dominates. Under these conditions, the majority of catabolic energy comes from light absorbed by bacteriochlorophylls (BChls) and carotenoids, pigments required for light harvesting and photosynthetic growth. Depending on the availability of light and/or oxygen, the metabolism will shift naturally from phototrophy towards fermentation **(3)** or respiration **(4)**. Under the latter conditions, catabolic energy is linked to substrate conversion. In the absence of both light and oxygen, fermentation **(3)** will prevail. When oxygen is present at high concentrations, the aerobic metabolism **(4)** will dominate regardless of the availability of light. The reason for this is the suppression of the synthesis of BChl and carotenoids under aerobic conditions (Yue et al., 2015), which causes a metabolic switch towards chemoheterotrophy or chemoautotrophy with O_2 as electron

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acceptor (*i.e.* respiration or nitrification) (Dubbs et al., 2000; Qian and Tabita, 1996). These growth modes have been used for the treatment of contaminated gas streams (removal of H_2S and CO_2 (Marín et al., 2019)) **(1)** and various wastewaters (Hülsen et al., 2018b). It is noted that mixed modes are possible, *e.g.* where part of the energy originates from chemical catabolism and part of the energy is generated from light, increasing the overall yields (*e.g.* mixed photo-fermentation) (Heinrich et al., 2016). Other than the applications described above, PPB have been used for soil remediation (Fan et al., 2012), polyphosphate accumulation (Hiraishi et al., 1991; Lai et al., 2017; Liang et al., 2010) or to monitor environmental stress (Kis et al., 2015).

With focus on carbon and resource recovery, anaerobic phototrophic energy generation (via photophosphorylation) has several advantages when compared with chemoheterotrophic growth. These include: (i) higher biomass yield, close to unity in chemical oxygen demand (COD) basis (*i.e.* one g COD biomass formed per g COD taken up, an advantage considering the biomass as value-added product, (Hädicke et al., 2011), and hence higher resource recovery efficiency; (ii) no aeration requirements and (iii) effective PPB selection and enrichment in non-sterile environments (Hülsen et al., 2016b, 2014). The latter is enabled by BChl a and b, which absorb near infrared (NIR) wavelengths (between 805 and 1035 nm), a capability almost exclusive to PPB (Madigan et al., 2011). In addition to this, an arsenal of more than 75 potential carotenoids (the particular composition is species dependent) extend the usable wavelength range of PPB to bands in the ultra violet (UV) and visible (VIS) light spectra (*e.g.* ~ 300 nm for rhodopin and ~ 500 nm for lycopene), besides providing photoprotection (Hartigan et al., 2002).

However, the light requirement is also a major drawback of phototrophic processes, which has been widely demonstrated in microalgae-based technology. Ensuring adequate light supply determines nutrient removal capacities as well as biomass production rates. The reactor must

then be designed for effective light delivery. To date, the vast majority of studies employing PPB have been done in artificially illuminated systems (174 out of 177 articles reviewed here). This option consumes substantial amounts of energy and has high capital costs due to the lamps required and the difficulty of delivering light to biomass growth regions, especially at large scale (needed for practical implementation). Because of these costs, phototrophic technologies are uneconomic compared with existing treatment technologies if only focusing on treatment goals. This also applies to the comparison with biotechnological applications, *e.g.* for high-rate production of valuable products with engineered microbes (Burgess et al., 2015). This leads to the main current challenge of PPB-based processes: the need to generate value-added products from waste streams to balance capital and operational costs. Similar to microalgal systems (which also face analogous challenges (Laurens et al., 2017; Ruiz et al., 2016)), potentially high value products from PPB include the biomass itself (*e.g.* used as fertilizer or animal feed) (Pikaar et al., 2018), proteins (as whole cell *i.e.* SCP, or extracted) (Hülsen et al., 2018a), molecular hydrogen (Ghosh et al., 2017; Hallenbeck and Liu, 2016), polyhydroxyalkanoates (PHA) (Fradinho et al., 2019) and high-value chemicals such as carotenoids, coenzyme Q10 (CoQ10) and 5-aminolevulinic acid (5-ALA) (Saejung and Ampornpat, 2019). Despite the increasing interest of PPB for biotechnological purposes, real-life applications (*i.e.* in industrially scalable processes) are scarce, limited to few niche applications (*e.g.* as probiotics, for water purification in aquaculture or as plant growth promotor (Qi et al., 2009)). To the knowledge of the authors, no companies exist applying PPB for resource recovery. Developments in widely researched microalgal PBR technology and in energy-saving light-emitting diodes (LEDs), paired with the shift towards resource recovery (Verstraete et al., 2009), have the potential to advance PPB-mediated innovative applications from the current embryonic technological state towards real world, full-scale applications. The critical issue to be addressed is identifying suitable products that can be

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efficiently generated from waste streams while providing effective waste/wastewater treatment. This will enable the implementation of comparatively higher-cost phototrophic technologies.

In this article, PPB-based environmental applications and potential products are reviewed to identify the major opportunities, challenges, research perspectives and development outlooks. In the first section, different waste streams and their potentials and limitations as substrates for PPB growth are presented. Crucial factors, including growth conditions and reactor operation, are also discussed. Secondly, the generation of value-added products within the PPB biomass is critically assessed. Thirdly, a comparison of the potential products, including volumetric productivities and economical aspects, is presented, highlighting implications for full-scale applications. Finally, the main challenges faced by PPB-based systems are discussed, and recommendations for future research and development are postulated.

2. Data collection and treatment

For this review, quantitative consolidated information was collected to identify performance limits and viability across the broad set of literature evaluated. A common set of normalisation factors was applied to compare studies across reactor types, substrates, feed conditions or inocula (including pure and mixed cultures, *etc.*). Quantitative and qualitative data were collected from 177 studies, obtaining a database consisting of 1,487 observations. Each observation included relevant information about a particular experiment (*e.g.* working conditions, inoculum used, reactor design, substrate fed or main outcomes). To produce a coherent database, different categories were defined for the reactors, the substrates and the inoculum source. The complete database and a list of the defined categories used in the study are given in Appendix A (Table A1). Omission of studies from this analysis was generally on the basis of missing main inputs and/or outputs, reported units not allowing to perform COD

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balances, or reactors with unclear redox conditions. The conversion factors and assumptions applied to standardise inputs to common basis for comparison can be found in Appendix B.

Statistical analyses were performed using the software R 3.5.0 (2019). ANOVA was used (on normally distributed, homogeneous variance populations) to assess significant differences, applying post-hoc Tukey HSD tests for comparisons. The validity of ANOVA was tested by normality analysis on source data (Shapiro-Wilk tests) and homogeneity of variance (Bartlett's tests). For non-normal, low count (n) tests, non-parametric tests were applied (using a Kruskal-Wallis test and Dunn's tests for pairwise comparisons). A significance threshold of $p = 0.05$ was applied.

The boxplots given provide the values for the lowest datum within 1.5·IQR (interquartile range) of the first quartile, the first quartile, the median, the third quartile and the highest datum within 1.5·IQR of the third quartile. Values below and above the lowest and highest data used for the boxplots were considered as outliers.

3. PPB production routes in waste streams

3.1. Impact of feed and inoculum

To grow photoheterotrophically, PPB require: a source of organic carbon, nutrients (mainly N and P) and light, specifically, NIR light above 800 nm. In synthetic media, the most commonly used nutrients have been NH_4^+-N and PO_4^{3-}-P, while other N-sources such as NO_3^--N or glutamate have been used to a lesser extent (Hülsen et al., 2014; Nagadomi et al., 2000; Tao et al., 2008). Common medium recipes have been provided by Ormerod *et al.* (1961) and Pfennig (1978). The tested carbon sources are much more diversified and include various simple organic compounds, such as volatile fatty acids (VFAs), simple sugars, alcohols and other organic acids (denoted as simple substrates). It is important to note that, thanks to the cyclic electron transport in anoxygenic photosynthesis, PPB do not necessarily require a

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dedicated electron donor or acceptor to grow. Only when a substrate is more reduced than the biomass (*e.g.* butyric acid), the substrate will serve as both, carbon source and electron donor to enable CO_2 fixation or other electron dissipation pathways (*e.g.* hydrogen production or storage of PHA or polyphosphate). In turn, CO_2 will be produced when the substrate is more oxidized than biomass (*e.g.* succinic acid, malic acid, *etc.*). Since organic carbon sources and inorganic donors are not required for ATP generation, PPB biomass yields on simple substrates can reach values up to 1.0 g COD·g $COD_{removed}^{-1}$ (Alloul et al., 2019; Hülsen et al., 2016b; Puyol et al., 2017). When the substrate is more reduced than the biomass, yields can be higher than 1.0 g C·g C^{-1} due to simultaneous fixation of CO_2. PPB biomass yields grown on different substrates (ranging between 0.5 and 1.0 g COD·g $COD_{removed}^{-1}$) are shown in Figure 2. The reason why some values are higher than 1.0 g COD·g $COD_{removed}^{-1}$ is not explained appropriately in the evaluated sources and is still speculative. Possible explanations include the slight overestimations of the produced biomass (*e.g.* by considering other solids as PPB biomass), underestimations of the supplied COD (*e.g.* not considering the COD of all the biodegradable organics in the media, such as yeast extract) and the presence of photosynthetic organisms oxidizing water to generate organics and O_2 (oxygenic photosynthesis). On the contrary, low yields can be explained by: (i) hydrogen production by PPB in nutrient limiting conditions, (ii) methane production if methanogens are present or (iii) fermentation or anaerobic oxidation to generate hydrogen by PPB or non-PPB organisms. These mechanisms are evidenced when assessing the yields by wastewater type, as shown in Figure 2, with organic acid feeds achieving yields close to 1.0 g COD·g $COD_{removed}^{-1}$, while sugary, digestate (where methanogens are present), and complex feeds achieving lower yields.

Although the utilization of synthetic media is an interesting and crucial approach for fundamental research, the substrate costs (*e.g.* 372-755 \$·t^{-1} (347-706 \$·t COD^{-1}) for acetic acid and 3,900–4,400 \$·t^{-1} (5,417-6,111 \$·t COD^{-1}) for malic acid (Mondala, 2015; Moscoviz

et al., 2018)) limit the economic large-scale production of PPB to the generation of very high value products, such as human food or food additives, an area which has not yet been developed with PPB (and probably cheaper using non-phototrophic alternatives). Instead, the growth of PPB in organic and nutrient rich waste streams has been recently proposed as a more economical alternative to synthetic feeds (Batstone et al., 2015). This approach provides wastewater treatment as well as nutrient and carbon recovery in the form of microbial biomass (Hülsen et al., 2018a, 2016b, 2014; Lu et al., 2019b). Median COD, nitrogen and phosphorus removals calculated from the literature are 76% (n = 205), 53% (n = 87) and 58% (n = 68), respectively, with optimal COD:N:P uptake ratios around 100:5:1, similar to ordinary aerobic heterotrophs and higher than other anaerobic biomass (100:1:0.1) (Figure A1) (Batstone et al., 2002). At these optimal COD:N:P ratios, removals efficiencies through assimilation of 97%, 92% and 94% for total COD, nitrogen and phosphorus, respectively, have been reported (Hülsen et al., 2016b). These results were obtained using a photo-anaerobic membrane bioreactor (PA-MBR) fed with domestic wastewater spiked with ethanol to adjust the COD:N:P ratio (at a solid retention time (SRT) of 2 d, a hydraulic retention time (HRT) of 0.5 d and an organic loading rate (OLR) of g 2.5 COD·L^{-1}·d^{-1}).

As shown in Figure 2, a range of agri-industrial and domestic wastewaters have been treated with PPB, obtaining biomass yields comparable to those achieved with synthetic media. Nevertheless, the existing literature dealing with PPB has predominantly used synthetic or axenic media and pure cultures. Out of the 177 reviewed studies, 70 studies deal with wastewater treatment and/or PPB biomass production. Amongst them, 73% applied pure cultures grown under axenic conditions (see Figure 3), mainly consisting of PNSB such as *Rhodobacter sphaeroides* and *Rhodopseudomonas palustris* (20 and 13% of the 70 studies). In addition, the vast majority of studies using wastewater as substrate used pure cultures as inocula and applied pre-treatments, such as autoclaving, centrifugation, filtering, heat

sterilization (pasteurization) and dilution (de Lima et al., 2011; Madukasi et al., 2010; Ponsano et al., 2008; Prasertsan et al., 1993; Wu et al., 2015). While this provides an indication of the general treatment capacities, it does not allow to systematically investigate and develop fundamental understanding on crucial aspects such as microbial competition, organic matter hydrolysis and syntrophic interactions. These factors may affect the actual removal performance and process stability over time, and reduce the validity of pure culture studies when applied to wastewater applications.

The remaining 27% of the studies presented in Figure 3 investigated the growth of PPB-enriched, mixed cultures under non-axenic conditions (with *Rhodopseudomonas* as predominant genus in most cases). These studies generally used NIR light (from LEDs or filtered full spectrum light) to enable effective PPB selection and enrichment in non-sterile media (Hülsen et al., 2014). This represents a more applicable approach to non-sterile wastewater treatment, which also considers microbial competition and syntrophic interactions (Hülsen et al., 2018a, 2016b). Particularly functional flanking clades include hydrolytic and acidogenic bacteria (among others), which enable solubilisation and fermentation of compounds which PPB may not be able to utilise directly (Ramsay and Pullammanappallil, 2001). This broadens the treatable wastewater spectrum. With an average value of 0.8 g $COD \cdot g\ COD^{-1}$, the photoheterotrophic biomass yields achieved using PPB-enriched cultures in non-sterile conditions are not significantly lower when compared to the yields achieved using pure cultures (Figure 4). In fact, the yields achieved with some pure cultures were lower than those achieved with PPB-enriched cultures, not being much higher than those commonly achieved in activated sludge systems (around 0.5 g $COD \cdot g\ COD_{removed}^{-1}$ (Henze et al., 2002)). As the yield is not substantially increased, the main value of using pure cultures is the improved growth rates, rather than improved yields. Treatment of wastewater sources requires mixed cultures. The impact of flanking communities on both function and product utility

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needs further investigation.

3.2. Reactor operation and configuration

Only a limited number of studies have attempted to optimize critical process parameters such HRT, SRT or OLR in continuous reactors with respect to performance indicators such as removal efficiency and biomass yield. These parameters are prerequisites for scale-up and will determine the size, performance and the feasibility of PPB-mediated wastewater treatment processes. Figure 5 shows the corresponding data (68% of the values correspond to PPB-enriched cultures). Both the OLR and the reactor configuration significantly affect the COD removal efficiencies. While continuous stirred-tank reactors (CSTRs) perform well at OLRs up to 2 g $COD·L^{-1}·d^{-1}$ (Figure 5), the limited biomass retention results in biomass washout and a decrease in the COD removal efficiencies at higher OLRs. Reactors providing biomass retention (*i.e.* PAnMBRs and photo-rotating biological contractors (PRBCs)) achieved high COD removal efficiencies even at high OLRs due to a reduced biomass wash-out. While sequencing batch reactors (SBRs) provide biomass retention via settling, their effectiveness depends on the actual settleability of the biomass, which is yet to be studied for PPB (Chitapornpan et al., 2012). Particularly promising results have been achieved using membranes for biomass retention (Hülsen et al., 2016b, 2016a). PAnMBRs illuminated with artificial NIR irradiance and using PPB-enriched cultures have achieved volumetric productivities of biomass up to 4.2 g $COD·L^{-1}·d^{-1}$, allowing COD, N and P recovery from wastewater (with removal efficiencies up to 93%, 89% and 44%, respectively) and with biomass light-energy yields around 59 g $COD_{biomass}·kWh^{-1}$ (Figure A2) (Hülsen et al., 2018b). In contrast, the productivities with CSTRs are below 0.8 g $COD·L^{-1}·d^{-1}$, with light-energy yields up to 3.4 g $COD_{biomass}·kWh^{-1}$ using tungsten lamps. It is interesting to note that most of the PBR configurations applied for PPB so far do not correspond to typical approaches applied for microalgae growth (*e.g.* open ponds, flat plate or tubular PBRs).

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Biomass retention, and hence high biomass levels, favour higher COD removal efficiencies due to the relatively low growth rates of PPB, which (together with light distribution) limits the specific removal rates and capacities (below 2.0 g $COD_{removed} \cdot g\ COD_{biomass}^{-1} \cdot d^{-1}$ and up to 2.5-2.6 g $COD_{removed} \cdot g\ COD_{biomass}^{-1} \cdot L^{-1}$ in most cases; see Figure A3). For example, specific growth rates in the range of 0.06-0.30 h^{-1} have been reported in pure cultures of *Rhodopseudomonas faecalis*, *Rhodopseudomonas palustris* and *Rhodopseudomonas gelatinosa*, at temperatures of 25-35 ℃ (Kim and Lee, 2000; Saejung and Ampornpat, 2019; Shipman et al., 1975). Growth rates for non-axenic PPB-enriched cultures are at the lower edge of this range, with values of 0.06-0.07 h^{-1} (25-40 ℃) (Hülsen et al., 2014; Kaewsuk et al., 2010). The faster growth rates in PPB pure cultures are a consequence of the favourable growth conditions caused by feeding pure/synthetic, sterile substrates (*i.e.* VFAs, malate or simple sugars) and the absence of competition. Nevertheless, real wastewaters rarely consist of pure substrates and OLRs from axenic trials cannot be accurately extrapolated to full-scale installations. This is shown in Figure 6, where significant differences between axenic and non-axenic biomass production rates exist for CSTRs and batch systems (with growth rates on axenic substrates being on average 3 and 8 times higher when compared to non-axenic conditions, respectively). Thus, it is evident that the design for both conditions will differ substantially, which underlines the need for studies using non-synthetic substrates. A similar problem has become clear in the scale up of algal systems, where indoor photosynthetic efficiencies were substantially higher when compared to those achieved in outdoor systems (Richardson et al., 2014). This has caused a considerable overestimation of the achievable areal productivities (extrapolated from lab-scale experiments and used *e.g.* in The Farm-level Algae Risk Model (Richardson et al., 2014)), which were not achievable in full-scale plants. This has contributed to a number of financial failures, particularly in the "green" biofuel industry (Reboredo et al., 2017).

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3.3. Economics of illumination

Another factor limiting the extrapolation of results in studies using axenic conditions and pure cultures is the type of light source applied. In most experiments, the light sources were either fluorescent, halogen or tungsten lamps or non-specific LEDs. Fluorescent, halogen and VIS-emitting LEDs are designed to minimize heat emission, reducing the output at NIR wavelengths. This leads to a very limited available light spectrum for PPB, which absorb predominantly above 805 nm (by BChl a and b). When growing PPB-enriched cultures in non-sterile substrates under these conditions, the competition with other phototrophs such as algae and cyanobacteria (absorbing predominantly at 400-700 nm) becomes an issue, particularly if the COD concentrations are not sufficient to support fast PPB growth and oxygen consumption (Suhaimi et al., 1987). There is very limited literature detailing this competition or the potential syntrophic behaviours that might appear, but it is likely that an imbalance will result in oxygen production by microalgae, leading to a suppression of the synthesis of BChl a and b and to inhibition of the photoheterotrophic metabolism of PPB (more information about the effect of oxygen on PPB growth can be found in Appendix C). To avoid this feedback loop, NIR output must be maximized, at least until a clear PPB dominance is established. Tungsten lamps emit a considerable fraction of NIR light and are suitable for PPB enrichment, but they are very inefficient. NIR-emitting LEDs maximize the NIR output and are energy efficient, thus representing the optimum artificial light source for PPB growth (see Figure A2). Compared to incandescent (*i.e.* tungsten) lamps, LEDs offer lower energy costs and NIR specificity, allowing for higher COD removal rates (Suwan et al., 2014). This is shown in Figure 7 (presenting the evolution of the PPB biomass energy yields with different light sources), where LEDs have much higher biomass energetic yields (between 10 and 100 times per kWh) than those achieved with other light sources (Hülsen et al., 2018b, 2018a, 2016a). NIR LEDs have a relatively high photon conversion efficiency of ~

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80% and an overall plug wall efficiency of 20-30% (Auf Der Maur et al., 2016). This is due to low forward voltages and optimal wavelengths for the mature phosphide emitter technology. In addition, NIR light has relatively low media attenuation due to its long wavelength. This means that modifications in emitter technology are unlikely to substantially improve reactor efficiencies. This is reflected in the data presented in Figure 7, where biomass energetic yields have peaked at 30-60 g COD·kWh^{-1}. The need of efficient light supply also represents an intrinsic barrier, as there is a compromise to be achieved between the surface/volume ratios in the reactors (determining the substrate load per illuminated area) and a sufficient COD concentration to support growth.

Assuming the maximum biomass energy yields achieved with LEDs so far (59 g COD·kWh^{-1}) and considering an energy price of \$0.0658 per kWh for industrial use (average in the United States in January 2019; higher in other countries) (U.S. Energy Information Administration (EIA), 2019), the energy cost to produce 1 kg of PPB results in 1.9 \$·kg$_{biomass}^{-1}$ (1.1 \$·kg$_{COD}^{-1}$). These illumination costs appear higher than the potential revenue from products that can be obtained from PPB (this will be further discussed in Section 5, where potential revenues for different products are given). In addition, this only considers operational illumination costs and does not include the capital required for lamps or any other expenses (*e.g.* lamp attachment), which increases the costs per illuminated square meter. It must be clarified that the maximum biomass energy yield provided (59 g COD·kWh^{-1}) corresponds to a purely practical value (*i.e.* energy consumed by the lamps).

The most obvious option to address this challenge is the utilization of solar light. So far, only three studies used sunlight to grow PPB (Adessi et al., 2012; Carlozzi et al., 2006; Carlozzi and Sacchi, 2001). Although the results from these studies suggest that outdoor PPB cultivation using tubular PBRs is technically feasible, all of them were carried out using pure PPB cultures and synthetic culture media. These outdoor studies aimed at producing PHA,

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biomass and hydrogen (Adessi et al., 2012; Carlozzi et al., 2006; Carlozzi and Sacchi, 2001). The main question remaining is how the value of these products can balance the capital and operational costs with reasonable amortisation. Can the costs be balanced if artificial media are used? How do these costs compare to those of other production processes? Is it cheaper to use wastewater? If so, is the process performance the same?

Although considerable research efforts have been dedicated in defined laboratory-scale systems, a huge research gap exists regarding the utilization of non-sterile substrates and future full-scale systems. From research on microalgae it is well known that reactors for phototrophic growth, especially closed systems, are capital intense and their feasibility ultimately depends on the product value and production rate. The different value-added products that can be obtained from PPB are discussed below.

4. Products derived from PPB cultivation

4.1. Microbial fertilizer, biofertilizer and liquid fertilizers

PPB, particularly PNSB, have recently gained attention due to their ability to produce and accumulate high-value compounds that are beneficial for plant growth (Sakarika et al., 2019). There are three routes to improve plant production using PPB and/or their by-products: (i) the use of the dried biomass as microbial fertiliser (*i.e.* source of plant nutrients), (ii) the use of active cells as biofertilizer (*i.e.* as active player in the rhizosphere microbiome), and (iii) the utilization of the liquid supernatant from a PPB culture, rich in 5-aminolevulinic acid (5-ALA). A recent review details all the benefits of PPB for plant production and the mechanisms involved (Sakarika et al., 2019). A synopsis of this approach is given here, with focus on PPB and by-products from wastewater.

The direct use of the PPB biomass as microbial fertilizer is facilitated due to their high biomass yields and nutrient content. Particularly relevant is P accumulation by PPB in the

form of polyphosphates, leading to higher P contents when compared with other microbial fertilizers (Sakarika et al., 2019). A number of studies have assessed the application of PPB biomass on the growth kinetics and yields of different crops, finding it a suitable fertilizer (Gamal-Eldin and Elbanna, 2011; Kantachote et al., 2016; Nunkaew et al., 2014a; Sakpirom et al., 2017; Wong et al., 2014; Zarezadeh et al., 2019). PPB biomass has been effectively applied as fertilizer for rice, rye grass, Chinese cabbage and citrus fruits cultivation, enhancing the yields to levels close to those achieved using commercial fertilizers (Gamal-Eldin and Elbanna, 2011; Kantachote et al., 2016; Kobayashi and Tchan, 1973; Sakpirom et al., 2017; Wong et al., 2014; Zarezadeh et al., 2019).

The application of PPB biomass as substitute of commercial fertilizers, such as diammonium phosphate (with current prices around 0.45 $\cdot kg^{-1}$ (Production & International Trade and Agricultural Services. International Fertilizer Association, 2017; Schnitkey, 2018)) represents a potential option to balance the production costs. In addition, if the final product achieves the classification of organic fertilizer (*i.e.* suitable for organic farming), the value could be increased to up to 0.8 $\cdot kg^{-1}$ (El-Haggar, 2007), a price that will ultimately depend on the PPB biomass grade and classification, which will be affected by the wastewater composition and local legislation.

5-ALA is produced extracellularly by PPB, particularly *Rhodopseudomonas palustris* (Kantha et al., 2010; Sakarika et al., 2019). 5-ALA is widely applied in the agricultural sector, often used as biodegradable herbicide, insecticide and as growth enhancer, promoting chlorophyll synthesis and crop yields and improving nutrient uptake (Kantha et al., 2010; Liu et al., 2016b; Sakarika et al., 2019). Concentrations up to 0.4 $g \cdot L^{-1}$ of 5-ALA have been detected in supernatants of PPB reactors, values high enough to allow direct application of the effluent as growth promotor (Kantha et al., 2010; Liu et al., 2016a, 2015). These concentrations could be further increased, as studies have found that the production of 5-ALA is enhanced by

submitting PPB to chemical stress, *e.g.* at high salinities, or in the presence of some transition metals like Zn or Cd (which would likely reduce the applicability of the effluent as irrigation water) (Kantha et al., 2010; Nunkaew et al., 2015, 2014a; Sakpirom et al., 2017). The substitution of commercial 5-ALA by biogenic 5-ALA from PPB, to be used as crop yield enhancer, might increase the value of the treated wastewater (Nunkaew et al., 2015, 2014b). Other applications of 5-ALA (*e.g.* cancer diagnosis and treatment) might further increase its value.

4.2. Single-cell protein

The terms single-cell protein (SCP) or microbial protein were introduced in 1968 to describe protein-rich foods derived from cells of microorganisms (*i.e.* bacteria, yeasts, fungi or algae) (Najafpour, 2007). While a wide variety of commercial SCP products are available for animal feed and even human food ingredients or supplements (FeedKind ®, UniPortein ®, Quorn ®, Solein ®, various suppliers of nutritional yeast such as SylPro ®, *Chlorella* and *Spirulina* biomass, *etc.*), currently no consumer products are made from PPB. It is unclear whether technological or non-technological innovation barriers are the reason for this, or just cold feet, as already in the 70-80s a number of studies discussed the possibility of producing SCP from PPB (Honda et al., 2006; Kobayashi and Tchan, 1973; Shipman et al., 1977, 1975). PPB cells are characterized by a high protein content and essential amino acid profiles suitable for nutrition, including high contents of the S-containing methionine and cysteine (generally low in other options, such as microalgae; see Figure A4 (Bleakley and Hayes, 2017)). Figure 8 compares the crude protein contents of a range of PPB species (pure and artificially mixed) axenically grown on synthetic substrates with those of PPB-enriched cultures grown in a non-sterile manner, including several wastewaters (*e.g.* abattoir processing effluents and domestic wastewater). In combination with the high biomass yields (Figure 2), the results (20-80% of the dry biomass weight corresponding to crude protein) confirm that non-axenic cultures have

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significantly higher or equal protein contents when compared with axenic, pure cultures. In addition, recent studies have confirmed that amino acid profiles from PPB-enriched cultures are comparable to that of common commercial protein sources (*i.e.* eggs, soybean or commercial fishmeal; see Figure A4).

The suitability of PPB biomass as nutritional SCP has been further underlined in several feeding trials, particularly as supplement in poultry (Ponsano et al., 2004) and commercial fish feed (though it has been much less extensively studied than microalgae or yeast). Fish feed trials include commercially relevant species such as Nile tilapia, marble goby, brine shrimp, barramundi, *Litopenaeus vannamei* and *Tor tambroides* (Alloul, 2019; Banerjee et al., 2000; Chowdhury et al., 2016; Getha et al., 1998; Loo et al., 2013; Shapawi et al., 2012). In addition, PPB have recently been applied as bulk substitute for fishmeal in barramundi feed trials. Increasing substitution resulted in a progressive decrease in fish weight gain and increased feed conversion ratios, but with no increase in mortality. However, substitution at up to 66% of fishmeal seemed commercially viable when compared to 0% substitution (Delamare-Deboutteville et al., 2019). This theoretically establishes a value for PPB biomass similar to fishmeal, which is currently around 1.5 $\cdot kg^{-1}$ or around 2.2 $\cdot kg^{-1}$ for fishmeal protein (Reuters, 2018a; The World Bank Group, 2019).

The production of protein-rich PPB biomass using wastewater as substrate therefore represents a possible approach to balance production costs (with costs from artificial illumination similar to the product value).

In terms of environmental footprint of the production of PPB SCP from wastewater, only one reference has been identified. Spiller *et al.* (2020) applied a sunlit PBR for treating anaerobically fermented potato wastewater. Compared to soybean meal, PPB was better for human health and ecosystems, yet consumed more resources (mainly energy), pointing out the need for renewable energy when producing SCP (electricity and heat).

4.3. Fine chemicals

4.3.1. Carotenoids

In addition to high protein contents, PPB contain an arsenal of carotenoids and BChls, which form part of their light harvesting complexes (Kuo et al., 2012). Carotenoids are organic pigments that are produced by plants, algae, bacteria, fungi and a few animals. Their commercial production as food supplement and in the chemical, cosmetics and medical industries is currently booming (Abu-Rezq et al., 2010; BCC Research, 2015; Ciriminna et al., 2016). In addition, they are used as supplements in aquaculture feeds, mainly due to their anti-oxidant and disease-preventing properties and their colouring capacity (Kuo et al., 2012; Liu et al., 2016c; Zhou et al., 2014). Prominent and valuable carotenoid examples are astaxanthin (2,500–7,000 $\cdot kg^{-1}$ (Panis and Carreon, 2016)), produced by microalgae (*e.g. Chlorella sp.*) and β-carotene (400-2,000 $\cdot kg^{-1}$ (Abu-Rezq et al., 2010)) and lycopene (around 6,000 $\cdot kg^{-1}$ (Ciriminna et al., 2016)), both generally extracted from vegetables. Among those, lycopene is found in significant amounts in PPB biomass (2-10 $mg \cdot g_{dry\ biomass}^{-1}$) (Su et al., 2018; Wang et al., 2012). These levels are on the border of commercial relevance, as current industrial production of natural-sourced lycopene relies on tomatoes (1-3 $mg \cdot g_{dry\ biomass}^{-1}$ assuming 95% water content (Olufemi et al., 2009)) and to a lesser extent in engineered *S. cerevisiae*, *B. trispora* and *E. coli* (24-73, 26-40 and 8-448 $mg \cdot g_{dry\ biomass}^{-1}$, respectively (Alper et al., 2006; Hernández-Almanza et al., 2016; Ma et al., 2019; Niu et al., 2017)).

The literature regarding individual carotenoids in PPB is limited, and the carotenoid contents have been largely expressed as total carotenoids, which are determined via spectrophotometric measurements based on extinction coefficients from absorption spectra of single carotenoids (Kopec et al., 2012; Rodriguez-Amaya and Kimura, 2004). This is particularly problematic in samples containing several carotenoids, each with different

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absorption spectra that also vary with the specific solvent used for extraction (Rodriguez-Amaya and Kimura, 2004). More detailed, individual carotenoids characterisation and quantification methods are based on HPLC-MS (and its derivates), which have been predominantly used in the biotech literature (Kopec et al., 2012; Rodriguez-Amaya and Kimura, 2004).

The carotenoid contents in PPB are summarized in Figure 9. The values vary between 0.5-13 $mg \cdot g_{dry\ biomass}^{-1}$ (Azad et al., 2001; Chitapornpan et al., 2013; Saejung and Apaiwong, 2015; Suwan et al., 2014; Urakami and Yoshida, 1993; H. Wang et al., 2017), with reported averages below 4.0 $mg \cdot g_{dry\ biomass}^{-1}$ and slightly higher contents in *Rhodopseudomonas faecalis* (Figure 9A). The reason for the different contents among PPB species is unknown, as comprehensive studies comparing the maximum carotenoid contents in different genera or species are missing. Factors such as carbon source (Figure 9B), light intensity (Zhou et al., 2014), light source (*i.e.* wavelength spectrum) (Kuo et al., 2012), salinity (H. Wang et al., 2017) and operational conditions (anaerobic, aerobic) (Liu et al., 2016c; Zhou et al., 2015), are likely to influence the overall carotenoid content more than the actual microbe. This might explain the lower average contents in PPB-enriched cultures (Figure 9A), although values of 6.5 and 6.8 $mg \cdot g_{dry\ biomass}^{-1}$ recently achieved in PPB biofilm systems, are similar to those obtained with pure cultures (Delamare-Deboutteville et al., 2019).

The highest carotenoid contents in PPB using simple substrates (Figure 9B) have been achieved with malate (5.2 $mg \cdot g_{dry\ biomass}^{-1}$ with outliers over 10 $mg \cdot g^{-1}$). Interestingly, the average carotenoid contents on domestic wastewater (determined using pure cultures) are comparable to those achieved with malate, and are significantly higher when compared to any other synthetic substrate.

The illuminance (*i.e.* luminous flux per unit area) also influences the total carotenoid content in PPB. Illuminance values up to an optimal range of 3,500-4,499 lx led to significantly

higher PPB carotenoids contents compared to lower ranges (Figure A5). However, values over 4,500 lx caused photoinhibition, which negatively affects the carotenoid content (Nath and Das, 2009; Shi and Yu, 2005; Zhou et al., 2014). In most cases, the illuminance only considers the VIS spectrum. Thus, when NIR is the single energy source, specific sensors must be used.

Although some carotenoids are naturally sourced, their global supply is currently dominated by synthetic products. The production of β-carotene exemplifies this, with a synthetic global market size around 1.5 and 3 times higher than those of vegetables/fruit and algae/fungi based extracts (Transparency Market Research, 2019). Interestingly, there is an ongoing controversy about the impact of synthetic carotenoids on human health, specifically β–carotene. Different studies have raised significant concerns about the effect and safety of synthetic, all-trans, β–carotene as human food supplement (Heinonen and Albanes, 1994; Patrick, 2000). Consequently, natural-sourced carotenoids are highly desired and have a substantial growth potential. In this context, PPB might serve as a potential natural source of carotenoids, although, to date, their application for industrial carotenoid production has been largely neglected, mainly due to the embryonic technological state and the lack of large-scale production systems.

A challenge of pure carotenoid production from microbial biomass is the high cost and complexity of the extraction/purification stages, usually based on energy-demanding solvent extraction processes (Bogacz-Radomska and Harasym, 2018), which further decreases the profit margins. Additionally, most traditional extraction processes involve the use of large quantities of at least one type of volatile organic solvent (*e.g.* acetone, methanol, ethanol or hexane) in direct contact with the biomass (Mondal, 2017; Rodriguez-Amaya and Kimura, 2004; Wang and Weller, 2006). These solvents are often hazardous and toxic, which creates environmental concerns, compromises the utilization of these high-value chemicals for human

consumption, and impacts the recovery of the residual biomass as by-product (Singh et al., 2015). Advanced green extraction processes are being developed to avoid these issues. These methods involve the use of less toxic and biodegradable alternatives such as extraction with ionic liquids, sub- and super-critical fluid extraction, or supercritical carbon-dioxide extraction (Passos et al., 2014; Singh et al., 2015). These extraction methods are more environmentally sustainable, often have lower costs, and generate a food-safe product.

A simple option to overcome the issues related to carotenoid extraction is the direct utilization of the carotenoids contained within the biomass. When a minimum carotenoid content in the biomass can be guaranteed, the protein-rich PPB biomass (i.e. grown on wastewater) can be used for purposes where both, protein and carotenoids are desired. Aquaculture feeds are a perfect example. In industrial fish farming, common protein meals (e.g. soybean, poultry by-products or blood and bone meals) are used as essential amino acid sources for fish rearing, while carotenoids (mainly astaxanthin and canthaxanthin) are added to enhance the salmonid health and their immune system, to increase the disease resistance and to give colour to the produced meat, which is an important product quality perception criterion (SKREDE and STOREBAKKEN, 1986; Waye et al., 2019). As an example, farmed salmon grown on commercial aqua feed (commonly mix of protein meals) do not have their characteristic pink-reddish colour because of a lack of a natural supply of carotenoids (e.g. krill) (Buttle et al., 2001). Therefore, the indirect carotenoid supply via PPB protein meal might substitute synthetic carotenoid in the feed. The authors note that the meat colouring effect of lycopene is questionable but that the value as anti-oxidant might still be interesting. In addition, carotenoid supply via whole PPB-cell feeding can serve as stabilizer for the easily oxidizable carotenoids (C. S. Boon et al., 2010). This approach could further increase the value of PPB biomass as fishmeal substitute, favouring the economics of this alternative.

4.3.2. Coenzyme Q10

PPB also contain CoQ10, an ubiquitous coenzyme that is present in both animals and bacteria, acting as an antioxidant and electron carrier (Yen and Shih, 2009; Zhu et al., 2017). CoQ10 is mainly used for medical and cosmetic purposes (Urakami and Yoshida, 1993; Zhu et al., 2017). Amongst the different options for CoQ10 production, microbial biosynthesis appears as a cost-effective approach that is already being applied commercially (with global prices around 500-600 $·kg^{-1} (Grand View Research; Market research and consulting, 2015)) (W. Lu et al., 2013; Urakami and Yoshida, 1993). PPB have been found to produce CoQ10 under phototrophic conditions, accumulating similar quantities compared to microbes currently used for CoQ10 production (contents of 0.2-9.3 mg·g$_{dry\ biomass}$$^{-1}$ for PPB and 0.8-3.3, 0.3-2.4 and 5.3-12 mg·g$_{dry\ biomass}$$^{-1}$ for yeasts, *E. coli* and *Rhizobium radiobacter*, respectively) (Corinne P. Cluis et al., 2012; Tian et al., 2010a; Urakami and Yoshida, 1993; Zhu et al., 2017). A summary of the production of CoQ10 by PPB is presented in Table 1.

Depending on the PPB species, the substrate fed and the reactor design, the CoQ10 contents and volumetric productivities vary considerably. Up until now, the main research efforts have been directed towards the selection of microbial species containing high amounts of CoQ10 using synthetic substrates. *Rhodobacter sphaeroides* and *Rhodospirillum rubrum* appear to be the most promising species, with contents up to 8.1 and 9.3 mg·g$_{dry\ biomass}$$^{-1}$, respectively (Kien et al., 2010; Tian et al., 2010b; Urakami and Yoshida, 1993). Gene deletion and overexpression have also been applied as strategies to further increase the CoQ10 contents (Kien et al., 2010; W. Lu et al., 2013; Zhu et al., 2017). Mutant PPB strains were reported to increase the CoQ10 concentrations in the media by 38% when compared with the wild type strain (W. Lu et al., 2013), obtaining up to 8.1 mg CoQ10·g$_{dry\ biomass}$$^{-1}$.

Compared with other organisms, only a few studies have dealt with the production of CoQ10 by PPB (specifically under anaerobic conditions). Nevertheless, CoQ10 represents yet another valuable product with the potential to increase the value of PPB biomass. The high cost of

CoQ10 and its expanding market (see Table 2) might allow the potential application of pure substrates (*e.g.* acetic acid) and axenic production (Urakami and Yoshida, 1993). As for carotenoids, the extraction and purification steps have to be considered. The combined value of proteins and non-extracted carotenoids and CoQ10 has not been assessed in dedicated feed studies, but might offer an interesting valorisation route.

4.4. Hydrogen and PHA production from nutrient-limited streams

PPB, like any other organism, cannot grow effectively in nutrient-deficient media (*e.g.* under lack of nitrogen, phosphorus, potassium, sulfur, *etc.*). In fact, if there is an excess of reducing power, PPB need to release excess electrons. If nutrients are present in sufficient amounts to allow growth, the main mechanism for electron dissipation (other than growth) is CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle. However, in growth limiting conditions the CBB cycle is not efficient and fast branches must be activated to maintain redox homeostasis. To do so, PPB can either dissipate this excess in the form of hydrogen or store it for further utilization in the form of PHA, which serves as carbon storage to be used for growth at a later stage, when sufficient nutrients become available (Fradinho et al., 2019; Ghosh et al., 2017; Hallenbeck and Liu, 2016). In both cases, hydrogen and PHA serve as electron sinks under growth limiting conditions. In the coming sections, both hydrogen and PHA as value-added products generated by PPB are further discussed.

4.4.1. Hydrogen production: application of PPB for energy recovery

Hydrogen is a commodity used worldwide for industrial purposes, mainly as a chemical commodity and energy carrier. Moreover, the current need of developing new sources of clean-renewable energy sources is driving the markets of novel energy carriers, with hydrogen being amongst the most promising options, with a compound annual growth rate (CAGR) of 15% (Persistence Market Research, 2018; Reuters, 2018b; Toledo-Alarcón et al., 2018).

Biological hydrogen production processes have gained attention in the last years, mainly due

to their claimed lower costs when compared to other processes (*e.g.* water electrolysis) and to the possibility of utilizing different wastes as substrates (Nikolaidis and Poullikkas, 2017; Reungsang et al., 2017). Nevertheless, biohydrogen production is currently not economically competitive when compared to processes based on fossil fuels, such as coal/biomass gasification or natural gas/biogas reforming (price of at least 2.6-2.8 $\cdot kg^{-1}$ *resp.* 1.3-2.3 $\cdot kg^{-1}$) (Moscoviz et al., 2018; Nikolaidis and Poullikkas, 2017). Consequently, the feasibility of biohydrogen production will depend on the market development, future CO_2-related policies and the prices of fossil fuels (Moscoviz et al., 2018). Despite being currently not cost effective, hydrogen production by PPB is included in this review due to its coverage in the literature and its potential feasibility in the future.

With over 80 scientific studies published in the last 10 years, hydrogen production has been the most widely researched PPB application. Despite not being a fermentation process, but rather an anaerobic oxidation using protons as electron acceptor and organic carbon as electron donor, hydrogen production by PPB is generally referred to as photofermentation (PF). PF occurs in PPB due to nitrogenase activity (related to nitrogen fixation), which is stimulated when soluble nitrogen sources are limited. This process is based on the consumption of energy generated via anoxygenic photosynthesis to reduce ferredoxin which, together with ATP, is used for proton reduction, resulting in the production of molecular hydrogen (Ghimire et al., 2015a). The ability to generate ATP from light makes PF interesting when compared to other alternatives. In contrast with dark fermentation (DF), hydrogen production via PF is not mechanistically linked to organic matter oxidation or catabolic processes. Therefore, PF avoids thermodynamic limitations such as the theoretical maximum yield of 4 mol H_2 per mol of hexose, with acetate as by-product, which cannot be further oxidised except at very low H_2 concentrations (Ghimire et al., 2015a). As a result, achievable hydrogen yields by PF are substantially higher when compared to DF (*i.e.* average values over

0.25 g $COD_{H2} \cdot$g COD_{fed}^{-1} *vs.* average yields of 0.11 g $COD_{H2} \cdot$g COD_{fed}^{-1} (Moscoviz et al., 2018)). In the case of PF, the average value of 0.25 g $COD_{H2} \cdot$g COD_{fed}^{-1} does not correspond to intrinsic biochemical limitations of the process (PF is not directly linked to growth or catabolism), but rather to non-optimal conditions (*e.g.* nutrients present to favour growth, competing PHA accumulation, non-fully degradable substrates or light limitations). This can be observed in Figure 10, where values up to 0.98 g $COD_{H2} \cdot$g COD_{fed}^{-1} are presented.

Among the organic compounds that PPB can utilize as carbon and electron sources for hydrogen production, simple organic acids such as acetic, butyric, lactic, malic and succinic acid, together with simple alcohols and sugars (regarded as simple substrates), have been the most widely tested (Barbosa et al., 2001; Chen et al., 2012; Melnicki et al., 2008; Tao et al., 2008; S. C. Wu et al., 2012). The hydrogen yields for different simple and complex substrates (*i.e.* solid hydrolysates and fermentation effluents) and different PPB inocula are presented in Figures 10A and 10B, respectively. As these figures show, pure PPB cultures studies, particularly with *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodopseudomonas palustris* represent most of the research carried out (65% of the data points in this study). In fact, most of the studies have been carried out using pure cultures and synthetic/sterile substrates (84% of the total studies dealing with hydrogen production via PF, see Figure A6). This leads to the same limitations described in Section 3, including substrates costs and competition with other microorganisms. Taking malic acid as an example (highest number of points in Figure 10A, n = 123), using it as PF substrate results in a monetary recovery of 1.5-1.9% (based on a malic acid price of 3.9-4.4 $\$\cdot kg^{-1}$ (Mondala, 2015) and a hydrogen yield of 0.26 g $COD \cdot$g COD_{fed}^{-1} (median for malate in Figure 10A), with an associated revenue of 2.9 $\$\cdot kg^{-1}$ of hydrogen (assuming an average price, see Table 2)). This underlines the necessity of using mixed cultures and non-axenic substrates. In addition, the available data show that similar performances can be reached when comparing PPB-enriched cultures to pure, axenic

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systems. For enriched cultures, the average hydrogen yields of 0.25 g COD·g COD_{fed}^{-1} are not significantly lower when compared to either of the most commonly used pure cultures.

As stated above, PF only occurs at low soluble nitrogen concentrations. This occurs because, besides electron dissipation, nitrogenases (enzymes responsible for hydrogen production during PF) are also responsible for reducing dinitrogen gas into ammonia-N. Therefore, in the presence of readily-available soluble nitrogen sources (*i.e.* ammonia), their activity is suppressed due to product-induced inhibition (Androga et al., 2011; Ghimire et al., 2015a). The inhibitory effect of ammonia-N can be observed in Figure 11, showing the hydrogen yields at different nitrogen concentrations. While glutamate leads to high yields at nitrogen concentrations of 100 mg $N·L^{-1}$ (up to 0.99 g COD·COD_{fed}^{-1}), values over 0.7 g COD·COD_{fed}^{-1} are only achievable at ammonia-N concentrations below 10-20 mg $N·L^{-1}$. Confirming the mechanistic suppression of nitrogenase activity at high ammonia-N concentrations, this compound inhibits hydrogen production regardless of the COD concentrations (*e.g.* oversupply). This is indicated by the similar hydrogen yields at varying COD:N feeding ratios (Figure A7). This reinforces the critical importance of the N-source used for growth to achieve efficient hydrogen production via PF (Kim et al., 2012, 2011; Sabourin-Provost and Hallenbeck, 2009; Tao et al., 2008; Vasiliadou et al., 2018). Glutamate can be considered as the preferred nitrogen source for biomass growth in PF, allowing high hydrogen yields (Ghosh et al., 2017; Vasiliadou et al., 2018). However, most waste streams that could potentially serve as substrates for hydrogen production (*i.e.* industrial wastewater, biowastes or fermentation effluents) usually contain high concentrations of ammonia-N (>> 10 mg $N·L^{-1}$) and have, in most cases, insufficient bioavailable COD to assimilate all the soluble N.

The use of nutrient-limited waste streams as source of water and carbon for hydrogen production appears as an option to reduce the substrate costs when compared to artificial media. Nevertheless, the low soluble N contents required to achieve acceptable hydrogen

yields limit the sources of potential waste streams considerably. In most cases, substantial dilution is required, increasing both the operational and capital costs (*e.g.* larger tanks) and compromising the overall process feasibility (Kim and Kim, 2013). Most of the literature has not considered these limitations and cost factors. While considerable research has been done in defined laboratory-scale systems, there is still a huge research gap regarding process upscaling and the utilization of non-sterile substrates.

Very few waste streams, including industrial sugar-rich effluents (Keskin and Hallenbeck, 2012) or residual glycerol (Chookaew et al., 2015), are low in N and rich in simple organics. To allow the valorisation of complex organic substrates (*e.g.* cheese whey wastewater (Rai et al., 2012), bread waste (Adessi et al., 2018), distillery wastewater (Laurinavichene et al., 2018), potato steam peels (Özgür et al., 2010a), corncob (Yang et al., 2010), molasses (Özgür et al., 2010b, 2010a), starch (Cheng et al., 2010, Su et al., 2009; Yokoi et al., 2001, 1998) and different agricultural residues (Jiang et al., 2016; Lu et al., 2018; Y. Wang et al., 2017; Zhang et al., 2018, 2014)) via PF, multi-stage systems have been developed, coupling a hydrolysis/acidification 1st stage prior to PF (Ding et al., 2017; Ghimire et al., 2015b; Kim and Kim, 2013; Lee et al., 2017; Xia et al., 2013). The coupling of DF and PF is the most widely researched approach, generally considering PF as a post-treatment to increase the hydrogen yield (Ghimire et al., 2016; Lee et al., 2002; Luongo et al., 2017; Montiel-Corona et al., 2015; Montiel Corona et al., 2017; Özgür et al., 2010b, 2010a; Ozmihci and Kargi, 2010; Rai et al., 2012, 2014; Rai and Singh, 2016; Su et al., 2010, 2009; Uyar et al., 2009; Zhang et al., 2018). Data show that the average PF yields obtained from simple substrates (with a value of 0.27 g $COD_{H2} \cdot$g COD_{fed}^{-1} from the collected data) or by DF coupled to PF (globally of 0.25 g $COD_{H2} \cdot$g COD_{fed}^{-1} (Moscoviz et al., 2018)) are significantly higher than those obtained in single-stage DF (0.11 g $COD_{H2} \cdot$g COD_{fed}^{-1}) (Moscoviz et al., 2018). In addition, coupling DF and PF might help to overcome another practical limitation of PF: the relatively low hydrogen

production rates. The maximum reported PF volumetric productivities in continuous reactors are 3.6 $L \cdot L^{-1} \cdot d^{-1}$ (using CSTRs), with mean values of 2.2 $L \cdot L^{-1} \cdot d^{-1}$ (Figure A8). This is far below the values reported for DF, with maximum and median productivities of 347 $L \cdot L^{-1} \cdot d^{-1}$ and 3.3 $L \cdot L^{-1} \cdot d^{-1}$, respectively (Moscoviz et al., 2018). This is a consequence of the lower biomass concentrations (limited by efficient light distribution) when compared with anaerobic fermenters, which limits the applicable OLR (Figure A8B). In consideration of the latter, it has been recently stated that the complete conversion of simple organic substrates (with low protein contents) that are not further oxidizable via DF is the main niche of application of PF processes (Lee et al., 2017).

Even when considering hydrogen production via PF as a potential process to increase the hydrogen yields from low-N waste streams after DF, a major factor to consider is the required energy supply as light. Considering a maximum illumination energy yield for hydrogen similar to the one for biomass, 59 g COD kWh^{-1} (which is very optimistic, as the biomass yields are considerably higher than those for hydrogen at similar illumination and working conditions), the energy recovery would be of only 29% (excluding any other energy input, such as substrates or mixing and assuming a hydrogen calorific value of 39.4 $kWh \cdot kg^{-1}$). Therefore, if a feasible PF application is to be developed, natural light must be used. Hydrogen production using sunlight has only been addressed in one study, using a pure culture of *Rhodopseudomonas palustris* fed with an artificial media consisting of malate and glutamate as carbon and nitrogen sources, respectively (Adessi et al., 2012). The industrial application of PF relies on a combination of relatively clean and inexpensive input materials, outdoor conditions and most likely enriched cultures to address the severe current economic shortfalls.

Further information on this topic can be found in scientific reviews focusing on different aspects, such as: key mechanisms in PF (McKinlay and Harwood, 2010), general advances on

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hydrogen production via PPB (Basak et al., 2014; Basak and Das, 2007; Dasgupta et al., 2010; Ghosh et al., 2017; Hallenbeck and Liu, 2016), photobioreactor design (Adessi and De Philippis, 2014), the use of genetically modified organisms (Kars and Gündüz, 2010), crop residues as substrate for PF (Lee et al., 2017) or coupling of DF and PF (Rai and Singh, 2016). The high number of studies focused on PF allowed to draw generalized conclusions regarding the optimal operational parameters to maximize the hydrogen yields. The influence of the most relevant parameters (*i.e.* pH, temperature, illuminance and substrate load) is discussed in Appendix D.

4.4.2. PHA (polyhydroxyalkanoates): production of bioplastics

When carbon is present in excess and nutrients are limited, PPB are able to produce PHA, such as poly(3-hydroxybutyrate) (PHB) and poly(3-hydroxy-butyrate-co-3-hydroxyvalerate) (PHBV), as storage compounds (Moscoviz et al., 2018). PHA production has been intensively researched in the last decades because of the numerous potential applications of these biopolymers, being precursors to biodegradable plastics, which can be applied in the fields of packaging, health care or cosmetics (Fradinho et al., 2016).

The majority of commercial PHA production is carried out by aerobic heterotrophic growth, either in pure or mixed cultures, on crop-derived, relatively pure, substrates (Chen, 2009; Moscoviz et al., 2018). Alternative substrates such as methane have been assessed on pure cultures of methanotrophs (Strong et al., 2016). These processes have achieved PHB contents of 0.5-0.9 g PHA$\cdot$g$_{dry\ biomass}^{-1}$ and yields of 17-32% in terms of substrate conversion (in weight) for aerobes and up to 1.1 g PHB$\cdot$g CH$_4^{-1}$ for methanotrophs (Chen, 2009; Strong et al., 2016). Despite these promising numbers, the price of PHA (1.9-4.4 \$$\cdot$kg^{-1}) is still relatively high when compared to petrochemical plastics (0.88-1.6 \$$\cdot$kg^{-1}), making PHB-based products non-competitive against petroleum-sourced plastics (Chen, 2009; Moscoviz et al., 2018; van den Oever et al., 2017). Nevertheless, new requirements to utilise bio-based and

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biodegradable polymers (such as the European Commission directive 2018/0172 on the reduction of the impact of certain plastic products on the environment (The European Parliament and Council, 2018) or the French law 2015-992 on Energy Transition for Green Growth (Assemblée nationale et Sénat Française, 2015)) call for the development of environment-friendly, biodegradable alternatives. PHA is projected to be the fastest growing bio-based and biodegradable polymer over the next 5 years, with a projected annual growth rate of 10-37% and a CAGR of 11% (2019) (European Bioplastics, 2019; Reportlinker, 2019). PPB can also accumulate considerable amounts of PHA, with contents ranging between 0.04-0.82 $g \cdot g_{dry\ biomass}^{-1}$. Figure 12 presents the PHA contents for different PPB inocula and substrates. As in previous sections, almost all research has been carried out using pure culture of the same aforementioned microorganisms. *Rhodobacter sphaeroides* achieved the highest PHB contents using axenic cultures (average of 0.26 $g \cdot g_{dry\ biomass}^{-1}$), significantly higher than those obtained using *Rhodopseudomonas palustris* (see Figure 12A). Interestingly, the highest average PHB contents (and the highest productivities, see Table S2) have been achieved with enriched cultures, not significantly different when compared to pure cultures of *Rhodobacter sphaeroides* (p-value of 0.999). Regarding the substrates used, acetic acid resulted in the highest PHB content, significantly higher than any other substrate (Figure 12B). A possible explanation for this might be that acetate can be easily converted into acetyl-CoA, which is the precursor of PHB formation (Karthikeyan et al., 2015). For most of the other organic substrates, acetyl-CoA production occurs via pyruvate dehydrogenase, while acetate conversion into acetyl-CoA occurs via acetyl-CoA synthase. This implies the release of reducing equivalents. Considering an excess of electrons already exists, the consumption of the less reduced substrates and the pathways dissipating the highest amounts of electrons will be favoured. Interestingly, very high PHB contents (up to 0.82 $g \cdot g_{dry\ biomass}^{-1}$) have also been recently reported when using DF effluents (rich in VFAs) as substrates (Table S2) (Ghimire et

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al., 2016; Luongo et al., 2017; Montiel-Corona et al., 2015; Montiel Corona et al., 2017). In fact, studies have shown that PPB can effectively remove COD from complex, non-sterile, nutrient-limited waste streams (*e.g.* molasses or VFA-rich wastewaters) while accumulating PHA, which can favour the economic feasibility of the process by removing the substrate costs, which are around 25% of the operating costs (Chen et al., 2012; Fradinho et al., 2019; Kim et al., 2012, 2011; Melnicki et al., 2009; Montiel-Corona et al., 2015; Montiel Corona et al., 2017; Özgür et al., 2010c).

Compared with aerobic microorganisms, PPB offer some advantages for PHA production: (i) higher overall PHA yields (g C-PHA·g C_{fed}^{-1}) are possible, (ii) the omission of aeration requirements; and (iii) effective enrichment of PHA accumulators via IR illumination. In addition, PPB biomass growth and PHA accumulation can be controlled by simply modifying the nutrient availability, without the need for feast-famine selection (as when using mixed heterotrophic cultures). The reason for this is that PPB can replenish reduced cofactors (*e.g.* NADH, NADPH) via PHA synthesis using ATP generated via photophosphorylation for carbon uptake under nutrient-limited conditions (Fradinho et al., 2016). This allows non-axenic PPB cultures to accumulate PHA in a permanent carbon feast regime, simplifying the process when compared to the 3-stage feast-famine operation used for mixed culture aerobic PHA accumulation (Fradinho et al., 2019). However, considering the illumination costs described in Section 3 (1.9 $·$kg_{biomass}^{-1}$; assuming that PHA synthesis and biomass growth have similar light requirements), the use of artificial light is not economically viable, requiring 1.1 $·$kg_{COD}^{-1}$ for PPB *vs.* around 0.28 $·$kg_{COD}^{-1}$ for aeration (assuming yields of 0.5 g COD·g COD^{-1} and that 50% of the total energy consumption is due to aeration (Guerrini et al., 2017; Henze et al., 2002)). This means that solar light is required to make PPB-PHA economically competitive with conventional production routes. Furthermore, the reported volumetric production rates (0.77 g·L^{-1}·d^{-1} with PPB; Table S2) are far lower when compared

to recent rates reported for aerobic heterotrophs (usually above 5-10 $g \cdot L^{-1} \cdot d^{-1}$ and up to 50-60 $g \cdot L^{-1} \cdot d^{-1}$ (Gahlawat, 2019; Valentino et al., 2017)). The lower rates achieved with PPB are mainly a consequence of light limitations, related to the light input (which is a natural constraint in outdoor systems) and/or the illuminated surface to volume ratio. These lower rates will generally result in higher reactor volumes and capital costs. Therefore, PHA production from PPB, while having some compelling advantages is not fundamentally lower cost than conventional aerobic production.

Another crucial point to be considered when producing PHA using PPB is the loss of electrons to hydrogen via PF. In COD-rich and N-limited streams both hydrogen and PHA synthesis occur simultaneously (Chen et al., 2012; Ghimire et al., 2016; Kim et al., 2012, 2011; Luongo et al., 2017; Melnicki et al., 2009; Montiel-Corona et al., 2015; Montiel Corona et al., 2017; Özgür et al., 2010c). As opposed to hydrogen production, PHA accumulation is not necessarily suppressed in the presence of ammonia-N. If other nutrients, such as phosphorus, magnesium or sulfur are limiting, PHA will also be produced, even at high ammonia-N concentrations (Melnicki et al., 2009; Mukhopadhyay et al., 2005; Padovani et al., 2016). An effective approach to limit hydrogen production and enhance PHA accumulation is to provide ammonium-N but limit other nutrients, thereby allowing PHA production while inhibiting nitrogenase activity. This might also broaden the variety of potential wastewaters to be used as substrates (*e.g.* after P, Mg or S precipitation (Melnicki et al., 2009)). Other options proposed to favour PHA accumulation include: using genetically modified organisms without poly(3-hydroxybutyrate) (PHB) synthase (Kim et al., 2011), using acetate as preferred substrate for PHA production (Kim et al., 2012; S. C. Wu et al., 2012) or pH control (Kim et al., 2011).

Downstream extraction of PHA from the biomass is not expected to be substantially different from heterotrophic aerobic production routes (which are also generally gram negative)

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(Valentino et al., 2017). Traditionally, this involved the use of halogenated solvents, and was the major cost of PHA production (over 50% of the total production costs (Chen et al., 2001)). However, new techniques are emerging such as temperature or osmotic disruption, the use of clean cellular solvents (such as supercritical CO_2) and the use of solvents such as acetone (in conjunction with temperature swing) (Jacquel et al., 2008). These have reduced the cost of PHA extraction so that the bioproduction step is the major cost, and have allowed the recovery of the residual biomass as a valuable by-product (instead of being contaminated with toxic solvents). As for carotenoids, the presence of PHA can increase the value of the biomass itself, avoiding the extraction step. For example, if the biomass is used as feed or feed supplement, PHA (without extraction) can increase its value by: (i) acting as biocontrol agent, reducing the need of antibiotic usage (Defoirdt et al., 2009), (ii) increasing the metabolizable energy content (Forni et al., 1999) and (iii) modulating and promoting the growth of gut flora (N. Boon et al., 2010). The dissociation of PHA in the gut into short chain fatty acids has been reported to inhibit the growth of pathogens such as *Escherichia coli* and *Salmonella typhimurium*, suggesting that it might add immune enhancing functions to the PPB product (Gowda and Shivakumar, 2019).

5. Towards viable PPB-based resource recovery and wastewater treatment

5.1. The need of generating added value: comparison of potential products

As described in the previous sections, the cultivation of phototrophic organisms has relatively high associated capital and operational costs. Even when using low value streams as substrate and natural light as energy source, PBRs used for PPB growth are more capital intense than their non-illuminated counterparts (*e.g.* SBRs or CSTRs). Raceway ponds or high rate algae ponds (HRAPs) have been applied for decades for microalgae growth (Oswald et al., 1957) and have been reported to be lower cost than closed algae PBRs (*i.e.* \$2.8-40 per illuminated

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m^2 *vs.* \$80-154 (Posten, 2009; Richardson et al., 2014; Slade and Bauen, 2013; Tredici et al., 2016; Young et al., 2017)). Some drawbacks of HRAPs include culture instabilities, biomass contamination, solid accumulation, large footprint, considerable evaporation losses and high harvesting costs (Buchanan et al., 2018; Flynn et al., 2017; Richardson et al., 2014). Both the advantages and disadvantages are valid for PPB growth in comparable systems. Pilot-scale PPB ponds are operational in Spain and Portugal, but there is still very limited literature about these units for PPB growth (Puyol et al., 2019). Regardless of the cultivation approach, to balance the higher capital investment and operational costs, high value-added products contained within, excreted by or synthetized from the PPB biomass must be recovered.

Considering this need for value-added products, the remaining question is which product is most favourable. This decision should consider several factors, including added-value, market size, regulatory restriction, competing processes, etc. The average contents of potential products within the PPB biomass are presented in Figure 13. The average crude protein content is significantly higher than any other potential product (0.52 g·g$_{\text{dry biomass}}^{-1}$). The methane production (estimated from the yields from anaerobic digestion of PPB biomass (Hülsen et al., 2020)) and the PHB content are not significantly different, with average values of 0.21 and 0.18 g·g$_{\text{dry biomass}}^{-1}$ respectively. The average CoQ10 and carotenoid contents are significantly lower than the rest (0.0038 and 0.0025 g·g$_{\text{dry biomass}}^{-1}$, respectively).

The product content within the biomass will directly affect the production rates (presented as volumetric production rates per reactor volume in Figure A9). The rates for the biomass itself are therefore the highest (average of 1.24 g·L^{-1}·d^{-1}), followed by proteins and by similar rates for methane and PHB (average values of 0.44, 0.26 and 0.19 g·L^{-1}·d^{-1}, respectively). A comparison with other technologies generating these products is challenging and depends on the substrates (*e.g.* wastewater), the reactor technology (high or low rate processes) and the microbes used (*e.g.* aerobic, anaerobic, *etc.*). Despite the higher biomass yields of PPB

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compared to aerobic or anaerobic systems (0.5-1.0 (Figure 2) *vs.* 0.5 and 0.2 g COD·g COD$_{removed}^{-1}$, respectively (Henze et al., 2002)), the biomass production rates are lower when compared to other options, which will result in higher capital costs. For high-rate anaerobic systems, OLRs up to 30 g COD·L^{-1}·d^{-1} can be reached (*e.g.* several full-scale internal circulation (IC) reactors). These are much higher than the typical values for PPB (2-4 g COD·L^{-1}·d^{-1}, see Figure 5), implying also drastically higher volumetric biomass production rates. This will also apply to the production rates of any other product contained within the biomass, such as SCP or PHA. In the case of methane (from PPB biomass), PPB growth will be the rate limiting step. However, due to the higher biomass yields, the methane yields from PPB per volumetric unit of treated wastewater will be higher while when compared to the yields from, *e.g.*, waste activated sludge (WAS) (assuming similar methane potentials (Hülsen et al., 2020)). Regarding PHA, the volumetric production rates from PPB are much lower compared to aerobic heterotrophs (grown on VFA) or methanotrophs, which can achieve above 5-10 g·L^{-1}·d^{-1} (Gahlawat, 2019). This limitation in rates also applies to hydrogen production with PPB, where average rates of 0.03 g·L^{-1}·d^{-1} are one order of magnitude lower when compared to DF (median values of 0.30 g·L^{-1}·d^{-1} (Moscoviz et al., 2018)) and drastically lower when compared to conventional production methods such as biomass gasification, electrolysis or biogas reforming (see Section 4.4). Concerning the production rates of CoQ10 and carotenoids, these compounds have the lowest production rates amongst all the internal cell products (0.0065, and 0.0038 g·L^{-1}·d^{-1}, respectively). These low rates will be a challenge for implementing the production of pure fine chemicals by PPB-based processes, as they might be outcompeted by existing industrial processes (*e.g.* chemical synthesis or biotechnological processes using genetically modified cultures and synthetic substrates). The production rates of extracellularly excreted 5-ALA are within the same order of magnitude of the CoQ10 and carotenoids (0.0031 g·L^{-1}·d^{-1}). Considering that productivities

of 0.73 g 5-ALA·L⁻¹·d⁻¹ have been achieved using recombinant fermenters, the production of pure 5-ALA via PPB will very likely be outcompeted in the market by other non-phototrophic biotechnological processes (Yang et al., 2016). However, the presence of 5-ALA in the effluent of a PPB treatment system might add value to the irrigation water (even when the value of 5-ALA in a complex matrix will be drastically reduced compared to the pure compound).

While a realisable process requires substantial whole of life costs, which will be different for the different products (and may still require substantial development), maximum achievable revenues from different PPB products can be calculated using the average contents and prices. The results (together with the market prices, market volumes and CAGRs for each product) are presented in Table 2. Although preliminary, these values indicate the maximum affordable production cost. Basically, when production costs are higher or close to the maximum potential revenue, the process is likely not feasible, either due to negative net present values or due to payback times that are too high to attract investment. In addition, competition with other production processes must also be considered, as it will determine a successful market uptake of the proposed technology.

The maximum potential revenue corresponds to the production of pure carotenoids from PPB (*e.g.* lycopene, resulting in an average PPB price of 9.25 $·kg⁻¹). With a potential value of 3.35 $·kg⁻¹, the production of 5-ALA is the second most valuable option, followed by CoQ10, with a potential value of 2.14 $·kg⁻¹. Nevertheless, the low contents and productivities of these compounds (see Table 2 and Figure A9) will complicate their production from PPB, surely requiring expensive extraction and purification processes. This will reduce the net revenue when compared to other products that omit or simplify these steps. Furthermore, the presented values are based on the assumptions that all the carotenoids/5-ALA/CoQ10 produced correspond to a pure/single compound and that all the content is recoverable. These

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numbers also ignore overestimations from inaccurate quantification methods. These assumptions will likely not be fulfilled in reality, which means that the revenues will be considerably reduced. These limitations might reduce this option to niche applications, as reported for microalgae, where few over-producing species are used only for carotenoid production (Sun et al., 2018).

Considering the direct application of bulk PPB biomass, its utilization as protein meal appears as a reasonable path forward. With prices around 1,500 $\$ \cdot t^{-1}$ (as of 5-09-2019), the substitution of fishmeal in aquaculture seems more profitable when compared to poultry meal (600-800 $\$ \cdot t^{-1}$) and soybean meal (around 400 $\$ \cdot t^{-1}$ (05-09-2019)) substitutions. The application of biomass as certified organic fertilizer is preferred over conventional fertilizer applications (*i.e.* values of 0.80 $\$ \cdot kg^{-1}$ *vs.* 0.45 $\$ \cdot kg^{-1}$). However, this possibility depends on the level of contaminants, which in turn depends on the growth media (*e.g.* wastewaters with high heavy metal content are likely to be unsuitable). The anaerobic digestion of PPB biomass to produce methane does not seem favourable at the current natural gas prices (0.54 $\$ \cdot kg^{-1}$, see Table 2). In addition, incomplete volatile solids (VS) destruction will result in sludge disposal costs, which has to be subtracted from the revenue. Considering the relatively low revenue from biogas and the low anaerobic digestibility of PPB biomass (around 50% VS destruction (Hülsen et al., 2020)), anaerobic digestion of PPB should only be considered when the utilization of the produced biomass for other purposes is not possible (*e.g.* when grown on domestic wastewater). This also applies to the production of hydrogen on nutrient-limited waste streams. The current energy and cost recoveries of hydrogen production (29% and 1.5-1.9% respectively, based on average H_2 yields and malic acid as substrate, see Section 4.4.1) seem prohibitive without major technological breakthroughs. In terms of PHA production, the prospect does not seem overly promising either. Based on an average PHA content of 0.18 $g \cdot g_{dry\ biomass}^{-1}$, the revenue per kg of PPB biomass is around 0.57 $\$ \cdot kg^{-1}$, which could be

potentially increased if higher PHA contents are maintained (up to 80% have been reported (Luongo et al., 2017)). The main challenge is the lower PHA production rates that can be achieved when compared to other alternatives. This is a consequence of the intrinsically limited light supply and the restricted means to optimize the illuminated surface to volume ratio of PBRs.

For bulk products, combinations of several components are possible. For example, PPB as fishmeal substitute might generate value as protein/amino acid source and might also have added value due to the carotenoids and even the PHA contents. In addition, the supernatant containing excreted 5-ALA can add further value as plant growth promoting irrigation water. Other options might include the carotenoid and or coenzyme Q10 extraction, where the residual biomass can be used as protein and/or PHA source.

It is important not to over-exaggerate the value of PPB and potential products, which has been a factor hindering realistic translation of algae technologies. For example values for algae biomass of 160 \$·kg^{-1} (Spolaore et al., 2006) or 250 €·kg^{-1} (Wijffels and Barbosa, 2010) have been proclaimed but never achieved except for specific niche markets.

5.2. Implications for industrial application of PPB-based processes

This study shows that, despite the economic challenges, PPB are promising candidates for carbon and nutrient recovery through assimilative growth, with a range of high-value products available. PPB-based technology is maturing from a developmental platform to an application-ready technology, with demonstration on a wide range of applicable wastewaters, needing translation to a realistic, economically viable process. The bulk product has been shown to have economic value, and there are multiple high-value constituents which may be extractable or enhance the value of the bulk product. PPB have also been shown to function at low wastewater temperatures (~10 °C), as well as under saline and even hypersaline conditions (Hülsen et al., 2019, 2016a). The fast and reliable selection and enrichment of

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ubiquitous PPB species present in the media is another major advantage that allows fast start-ups using PPB-enriched cultures for virtually any waste stream. This selection also affects the competitiveness of PPB-enriched cultures over time, where relative abundances of PPB can be over 60%, consistently for all types of non-sterile substrates (Hülsen et al., 2019, 2018a, 2016b). This is a major difference compared with microalgae-based processes, where increased wastewater strength (particularly at high COD concentrations) results in the establishment of ALBAZOD, with potential dominance of aerobic heterotrophs and thus reduced microalgae abundances (Hülsen et al., 2018b). In addition, the absence of O_2 as photosynthetic by-product and the photoheterotrophic metabolism remove the requirement to supply gaseous CO_2 and to remove O_2, particularly in closed systems (it should be noted that O_2 removal in algal PBRs is not an issue when feeding wastewater). Mixing is only required for substrate transfer, which substantially reduces costs. In addition, the HRT and SRT can be as low as 1-3 days, which substantially reduces the reactor volume when compared to microalgal processes (HRT and SRT of 5-10 days (Jorquera et al., 2010)). The advantages of PPB over algae are further reinforced by the generally lower light intensity requirements (*e.g.* < 50 W·m^{-2} (Chitapornpan et al., 2013; Suwan et al., 2014) *vs.* 100-200 W·m^{-2} (Gordon and Polle, 2007) and potentially higher photon conversion efficiencies (*e.g.* 6-8% *vs.* < 5% (Posten and Schaub, 2009)). With photosynthetic efficiencies of 4.6-6.0% (Zhu et al., 2010), photosynthetic plants are also outcompeted by PPB. Regardless of this, where artificial light input is used, the illumination cost (on power alone) makes most bulk products uneconomic. This means that solar radiation (with filters or wavelength shifting compounds – fluorescent phosphors are ideally suited for this) is currently required to enable economic illumination, which also eliminates the capital cost of artificial illumination.

There is a very limited number of studies that can be extrapolated to full-scale scale applications, with the bulk of the literature being in the lab using pure or defined cultures. The

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lack of data under outdoor conditions is particularly relevant. The application of natural light drastically increases the illumination intensity while reducing illumination times, but the effects on the treatment performances and the product yields are basically unknown (Hülsen et al., 2016b). The outdoor reactor design and operation might be particularly challenging at full sunlight, which can result in photoinhibition and microalgae/heterotrophic bacteria predominance. Filtered sunlight might solve these issues (several potential filters are commercially available; *e.g.* Lee filter, ND 1.2 299 (Hülsen et al., 2014)). In addition, efficiency losses in large-scale installations (*e.g.* due to reduced photosynthetic efficiencies, mixing, *etc.*) and seasonal effects (*e.g.* temperature variation, weather, *etc.*) are yet to be determined. Decades of research on microalgae for mainstream nutrient recovery could not translate the lab-scale performances into the field without major losses, an issue that needs to be avoided with PPB (Akkerman et al., 2002).

In this context, it is crucial to achieve reasonable production rates to enable product generation, ideally close to laboratory results. However, high rates alone will not suffice. The products, especially those dedicated for feed applications, have to be produced at a consistent quality throughout the year. Varying protein content (and carotenoids, PHA, *etc.*), high ash content and the presence of pathogens and/or high heavy metal contents, are highly relevant for a potential buyer (*e.g.* feed manufacturer) and will ultimately determine the value of the biomass. In terms of producing animal feeds, the European Food Safety Agency stipulates as a first criterion that the end product must be constant in composition. Assuming that consistent quality can be achieved, the next hurdle, especially when wastewater is the substrate, is the harvesting and drying of the biomass. Experience with SCP production and harvesting has shown that the optimal route is to centrifuge microbial biomass to obtain a slurry with minimum 10% of dry matter content. Subsequently, the slurry is pasteurized and spray dried to a minimum 85% dry matter. Practice has shown that this line of harvesting

(centrifugation followed by pasteurization and spray drying) imposes a cost of around 400 $·$t_{dry \ matter}^{-1}$ obtained, including both capital and operational expenses (personal communication with Avecom NV, Belgium). A final consideration relates to the legislation. In the European Union, the presence of faecal matter in the input of the production reactor excludes all uses as animal feed (EFSA regulation). However, the other regulatory demands for SCP destined for animal feed (provided the input material is free of faecal contaminants), such as is the case for many side streams in the food and feed industry, can be potentially met using PPB biomass. Indeed, pasteurization and spray drying by themselves decrease the presence of hygienic indicator species (*e.g. E. coli*) and spore formers to the levels stipulated by normal feed legislation. The authors are not aware of any PPB species with a current status of directly acceptable food or feed.

Besides the generated products, the removal of COD, nitrogen and phosphorus from wastewater adds additional savings due to lower discharge costs, which can be estimated based on volumetric biomass production rates (Figure 6). Savings from simultaneous removal of COD, nitrogen and phosphorus might be more valuable than the product itself, depending on the local trade waste fees. In Queensland (Australia), the discharge fees to sewer for a large producer (*i.e.* over 10 m^3 per day, 0.67 $·$kg BOD^{-1}, 1.51 $·$kg N^{-1} and 1.20 $·$kg P^{-1} (Urban Utilities, 2019), can result in savings per kg of PPB biomass produced of around $0.7 (based on biomass with a COD/VS ratio of 1.7, a COD/BOD equivalent of 2 and 10% N and 1% P contents). Considering European countries, the savings could be of $0.2-1.9 per kg of PPB biomass (taking Germany, Denmark, France and Spain as examples) (ECOTEC, CESAM, CLM, University of Gothenburg, 2001; Rahola et al., 2009). With these values in mind and considering that commercially available removal technologies are more effective and cheaper for removing COD (*e.g.* IC and UASB reactors, AnMBRs, CAS or HRAS), nitrogen (*e.g.* biological nutrient removal, anammox) and phosphorus (*e.g.* Crystalactor or

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struvite precipitation) when compared to phototrophic systems, the utilization of PPB-based processes must focus on its main advantages: (i) simultaneous recovery of COD, nitrogen and phosphorus (avoiding different treatment steps to alternate redox conditions), (ii) higher biomass yields when compared with those typically obtained in aerobic/anaerobic treatment processes (0.5-1.0 (Figure 2) *vs.* around 0.5 and 0.2 g COD·g $COD_{removed}^{-1}$, respectively (Henze et al., 2002)), (iii) higher nutrient content in the produced biomass, with COD:N:P ratios up to 100:11:1.9-100:14:2.2 (Hülsen et al., 2018a) and (iv) crude protein contents in excess of 60% dry weight (which is similar to aerobic heterotrophs, but higher when considering the biomass yield). Hence, PPB-based technology offers superior potential in terms of resource recovery efficiencies. To be successful, resource recovery technologies need to be economically comparable with existing technologies, while providing additional benefits. This trend can be assisted, but not broadly enabled, by legislation, including imposing mandatory P recovery (Austria, Switzerland or Germany) or source-sorted waste valorisation instead of its disposal (France, LOI n° 2015-992).

5.3. Perspectives and research and development outlooks

To advance the generalized application of PPB-mediated processes from their current embryonic state, the technology has to move outdoors, using enriched cultures grown on waste streams. Working outdoors, the impact of different cultivation options (*e.g.* different PBR configurations or high rate ponds), varying natural weather conditions and diverse substrate characteristics on the actual wastewater treatment capacity, the production costs per ton of biomass, or the quality and consistency of the desired product have to be determined. Ideally, long-term studies should be performed, accounting for weather variations during the different seasons. These systems also need to study basic parameters such as HRT, SRT and OLR, which determine the volume of the plant, as well as the volumetric productivities and removal rates. The latter (in terms of COD, nitrogen, phosphorus and solids treatment

capacity) is particularly relevant when considering the critical contribution of discharge savings to the economic feasibility of PPB applications. Globally, the reactor configuration, the PPB production costs, the discharge savings and the product revenue will determine the real-life feasibility of the process.

Until now, the literature does not converge towards a preferred PBR design for PPB. So far, the energy demand in all publications is prohibitive and scale-up has not been addressed. These energy requirements include reported illumination as well as mixing costs, but ignore harvesting or downstream processing. Recently determined harvesting and dewatering costs for microalgae cultivation in open ponds are between 0.3 and 2.2 $·kg^{-1} (depending on the cell density; 0.05 – 15% dry matter), with an anticipated energy consumption around 4.5 kWh·kg^{-1} (Fasaei et al., 2018). Drying costs in the order of 0.1-0.3 $·kg^{-1} have to be added (Mujumdar, 2014). In principle, configurations used for microalgae growth are feasible and operational costs might be, at least partly, transferrable. However, potential costs savings due to anaerobic conditions and photoheterotrophic growth without CO_2 requirements, likely enable alternative, cheaper mixing methods. This has to be specifically determined for each concept as mixing energies vary drastically between options (*e.g.* ponds, flat plate reactors, CSTRs, tubular PBRs or AnMBRs). The optimal light path is also yet to be determined and will significantly affect the volumetric removal and biomass production rates. The reduced penetration of IR relative to UV-VIS will be of major relevance in this regard. The potential oxygen transfer in open systems with large air-exposed surfaces (*e.g.* open ponds) will also have to be assessed, as this might disrupt anaerobic conditions (particularly if paddle-wheels are used for mixing).

The wastewater treatment performance will also be significantly affected by the reactor configuration, not only in terms of biological activity, but also in terms of physical separations. For example, systems retaining solids (*e.g.* AnMBRs, biofilm-based reactors or

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reactors with solid separation and recycle) will favour biomass retention and will generate clearer effluents, ideally free of suspended solids. In addition, this will aid the biomass harvesting process, producing a more concentrated biomass-rich stream. On the other hand, systems relying on physical separation of solids will generally not differentiate between the type of solids collected, which will eventually affect the quality of the harvested product (particularly in waste streams with high solid concentrations). Biofilm systems might increase selectivity via separate VS collection, possibly improving also product consistency. To tackle these challenges, different options for solid removal and biomass harvesting (*e.g.* sedimentation, flocculation, centrifugation, filtration or membrane reactors) must be further tested.

Straight-forward solutions to avoid product contamination due to harvesting of undesirable solids are using solid-free wastewaters or pretreating the wastes (*e.g.* by physical solid removals or via acidification). A pre-fermentation step can serve for this purpose, expanding also potential waste streams that could be valorised using PPB (such as easily degradable solid wastes (Capson-Tojo et al., 2015)) and possibly reducing the HRTs via feeding of directly assimilable organic acids. The availability of VFAs substantially affects the size of a potential plant, as the specific uptake rates are much higher when compared to other process such as hydrolysis, being the latter the rate limiting step (2.4 g $COD_{acetate} \cdot gCOD_{biomass}^{-1} \cdot d^{-1}$ (Puyol et al., 2017) *vs.* below 1.0 d^{-1} (Batstone et al., 2002)). Therefore, the pre-fermentation step can also lead to reductions of the reactor volume by enhancing the bioavailability of organic carbon and nutrients, hence increasing the volumetric removal rates.

Regarding the products to be generated, critical amounts of PPB biomass of the order of at least thousands of tons per year of dry matter have to be produced to estimate the annual revenues. Pot and field trials experiments must be carried out to provide insight on the value as fertilizer, while animal feed trials are needed to elucidate the value of the biomass as feed

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or feed component. As aforementioned, product consistency is key, especially for applications that rely on direct use of the biomass (*e.g.* fertilizer or SCP for feed). The combination of biomass constituents such as PHA, carotenoids and proteins should be tested to increase the overall value, moving beyond sole protein substitution. It must be considered that potential genetic engineered strains for the production of various fine chemicals (*e.g.* carotenoids or CoQ10) on food grade substrates are not considered in this document. Due to the light limitations, several other mediators (*e.g.* aerobes) are likely to be more profitable for this purpose. In addition, it is advisable to start from substrates which are relative clean in terms of faecal contaminants so that a direct line to feed production, acceptable to the consumer and fulfilling regulations, can be set up. In this context, the local/national legislation for any type of application has to be reviewed. This will enable a directed choice of substrates for specific applications, affecting also the real value of the final product.

A final comment must be made regarding the land surface requirements of potential PPB-based systems. As with any other phototrophic process, PPB-based processes will require a considerably larger surface when compared to non-irradiated wastewater treatment technologies. As an example, the land required for an HRAP treating 1000 $m^3 \cdot d^{-1}$ was estimated to be over 80 times higher compared to an equivalent conventional activated sludge system (Robles et al., 2020b). In this regard, PPB technology would be limited to small and decentralized processes. Further research should be done to precisely estimate the land requirements for various reactor configurations (*i.e.* PBRs *vs.* open ponds).

6. Conclusions

PPB-based processes represent a promising alternative to conventional concepts for resource recovery. The applicability of PPB for domestic and various industrial sectors has major advantages, and the recovery of a whole range of potentially valuable components can

combine discharge savings with substantial revenue streams. Particularly, the route of upgrading quality side-streams by means of PPB-based processes to animal feed offers potential. Higher value products are required to make resource recovery economically feasible, and PPB is no different in this regard. Delivery of solar light offers the potential to substantially reduce operational costs. This technology is a not a niche application and while its current embryonic (or infant) state requires further investment and development, it can benefit greatly from 60 years of microalgal and SCP-based technology developments. To advance its readiness level, the technology must leave the lab to translate the existing knowledge into real revenue/costs inventories that consider all the relevant factors and steps, including cultivation method, water treatment performance, product value, biomass harvesting, downstream processing and overall capital and operational costs. This has to happen on-site with potential early adopters and in multidisciplinary teams, including wastewater producers, product manufacturers and end users. An exciting period lies ahead, which might enable PPB-based technology to fulfil its potential to improve carbon and nutrient recovery efficiencies, bringing our waste and wastewater management systems one step closer to a sustainable and circular society.

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Author contributions

G. Capson-Tojo and T. Hülsen conceived the initial concept, wrote the article and defined the overall structure. G. Capson-Tojo, M. Grassino and A. Ghimire collected the data. G. Capson-Tojo carried out data treatment and plotting. S. E. Vlaeminck and W. Verstraete contributed actively to the single-cell protein section. D. Puyol and A. Ghimire contributed to the hydrogen section. M. Grassino contributed to the carotenoid section. A. Oehmen contributed to the PHA section. D. J. Batstone, S. E. Vlaeminck, D. Puyol, W. Verstraete, R. Kleerebezem, I. Pikaar and J. M. Lema reviewed the article and contributed to the overall writing, coherence and context of the review.

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Abbreviations

AMBR	Aerobic membrane reactor
AnMBR	Anaerobic membrane bioreactor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BChl	Bacteriochlorophylls
BOD	Biochemical oxygen demand
CAGR	Compound annual growth rate
CAS	Conventional activated sludge
CBB	Calvin-Benson-Bassham
COD	Chemical oxygen demand
CoQ10	Coenzyme Q10
CSTR	Continuous stirred-tank reactor
DF	Dark fermentation
DO	Dissolved oxygen
HRAS	High-rate activated sludge
HRT	Hydraulic retention time
HSD	Honestly significant difference
IC	Internal circulation
IQR	Interquartile range
LED	Light-emitting diode
MSBR	Membrane sequencing batch reactor

n	Number of independent members of a statistical population/sample
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NIR	Near infrared
OLR	Organic loading rate
ORP	Oxidation reduction potential
PAnMBR	Photo-anaerobic membrane bioreactor
PBR	Photo-bioreactor
PHA	Polyhydroxyalkanoate
PHB	Poly(3-hydroxybutyrate)
PHBV	Poly(3-hydroxy-butyrate-co-3-hydroxy valerate)
PF	Photofermentation
PNSB	Purple non-sulfur bacteria
PPB	Purple phototrophic bacteria
PRBC	Photorotating biological contractor
PSB	Purple sulfur bacteria
SBR	Sequencing batch reactor
SCP	Single cell protein
STP	Standard temperature and pressure
SRT	Solid retention time
TCA	Tricarboxylic acid
TCOD	Total chemical oxygen demand
TS	Total solids
UASB	Up-flow anaerobic sludge blanket
UV	Ultra violet

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VFA	Volatile fatty acid
VIS	Visible
VSS	Volatile suspended solids
VS	Volatile solids
VS_{frac}	Organic fraction of total solids
WW	Wastewater
5-ALA	5-Animolevulinic acid

Figure and table captions

Figure 1. Simplified representations of the main metabolic modes of PPB, (A) structured according to energy and carbon sources and electron acceptors and (B) rendered in a Venn diagram including the dominant mode under the presence/absence of organic matter, oxygen or light. ED stands for electron donor and COD for chemical oxygen demand.

Figure 2. Anaerobic photoheterotrophic biomass yields on different substrates. "Synthetic media" (green) comprise pure, well-defined, synthetic substrates containing VFAs, simple sugars, alcohols and other organic acids. "Waste streams" (red) correspond to heterogeneous substrates. The blue dots represent mean values. Only the substrates with three or more independent values (n ≥ 3) are presented. COD stands for chemical oxygen demand, WW for wastewater and VFA for volatile fatty acid.

Figure 3. Inocula used for biomass production and/or wastewater treatment using PPB. The numbers represent the percentages of a total of 70 studies. The data from "PPB-enriched culture" corresponds to enriched cultures grown on non-sterile, complex media. "Mixed PPB pure culture" corresponds to two or more combined pure PPB species.

Figure 4. Anaerobic photoheterotrophic biomass yields reported in the literature for axenic, pure inocula (white) and non-sterile, PPB-enriched cultures (red; dominated mostly by the genera *Rhodopseudomonas* and *Rhodobacter*). The blue dots represent mean values. Only the inocula with three or more independent values (n ≥ 3) are presented. COD stands for chemical oxygen demand and PPB for purple phototrophic bacteria.

Figure 5. Anaerobic photoheterotrophic COD removal efficiencies at increasing OLRs for different reactor configurations: photo-anaerobic membrane bioreactor (PAnMBR; ●), open-PAnMBR (◍), photorotating biological contractor (PRBC; △), overflow photobioreactor (PBR; ▲), membrane sequencing batch reactor (MSBR; ◇), sequencing batch reactor (SBR; ◆) and continuous stirred-tank reactor (CSTR; ■). COD stands for chemical oxygen demand and OLR for organic loading rate.

Figure 6. Anaerobic photoheterotrophic biomass production rates for different reactor configurations. Axenic (red) and non-axenic (blue) studies are presented separately. Only the reactor configurations with three or more independent values (n ≥ 3) are presented. COD stands for chemical oxygen demand, CSTR for continuous-stirred tank reactor, PAnMBR for

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photo-anaerobic membrane bioreactor, PBR for photobioreactor and SBR for sequencing batch reactor.

Figure 7. Evolution of the PPB anaerobic photoheterotrophic biomass energy yields obtained in processes illuminated with different light sources: not specified (■), fluorescent (◈), halogen (◯), tungsten (●) and light-emitting diode (LED; ▲).

Figure 8. Crude protein contents in photoheterotrophically-grown PPB biomass reported in the literature for axenic, pure inocula (white) and non-sterile, PPB-enriched cultures (red; dominated mostly by the genera *Rhodopseudomonas* and *Rhodobacter*). The blue dots represent mean values. Only the inocula with three or more independent values (n ≥ 3) are presented. PPB stands for purple phototrophic bacteria.

Figure 9. Carotenoid contents in the PPB biomass for (A) axenic, pure inocula (white) and non-sterile, PPB-enriched cultures (red; dominated mostly by the genera *Rhodopseudomonas*). and (B) substrates. The blue dots represent mean values. Only the conditions with three or more independent values (n ≥ 3) are presented. PPB stands for purple phototrophic bacteria and WW for wastewater.

Figure 10. Hydrogen yields achieved with PF using (A) simple substrates (green) and complex substrates (red) and (B) axenic, pure inocula (white) and non-sterile, PPB-enriched cultures (red; dominated mostly by *Rhodopseudomonas palustris*). The blue dots represent mean values. Only the categories with three or more independent values (n ≥ 3) are presented. COD stands for chemical oxygen demand, VFA for volatile fatty acid, PPB for purple phototrophic bacteria, PF for photofermentation and PHB for poly(3-hydroxybutyrate).

Figure 11. Hydrogen yields achieved with PF at increasing initial/influent nitrogen concentrations. The different compounds used as N-source for PPB growth are presented separately. PF stands for photofermentation, COD for chemical oxygen demand and PPB for purple phototrophic bacteria.

Figure 12. PHA contents for (A) axenic, pure inocula (white) and non-sterile, PPB-enriched cultures (red; unknown predominant genus/species) and (B) different substrates. The blue dots represent mean values. Only the categories with three or more independent values (n ≥ 3) are presented. PHB stands for poly(3-hydroxybutyrate), PPB for purple phototrophic bacteria and VFA for volatile fatty acid.

Figure 13. Contents of different potential products within the PPB biomass. Methane refers to the methane yields produced via anaerobic digestion (Hülsen et al., 2020). The coloured squares represent the mean values achieved with different reactor configurations: batch (■), sequencing batch reactor (SBR; ■), fed-batch (■), fed-batch attached (■), overflow photobioreactor (PBR; ■), tubular PBR (■), photo-anaerobic membrane bioreactor (PAnMBR; ■) and continuous stirred-tank reactor (CSTR; ■). PHB stands for poly(3-hydroxybutyrate) and PPB for purple phototrophic bacteria.

Table 1. Main results regarding coenzyme Q10 production using PPB. CSTR stands for continuous stirred tank reactor, nr stands "non-reported", and PPB for purple phototrophic bacteria.

Table 2. Current market prices and volumes of the different potential products. The potential revenue due to the content of each product within the PPB biomass is also presented. PPB stands for purple phototrophic bacteria, SCP for single-cell protein, 5-ALA for 5-Aminolevulinic acid and PHA for polyhydroxyalkanoate.

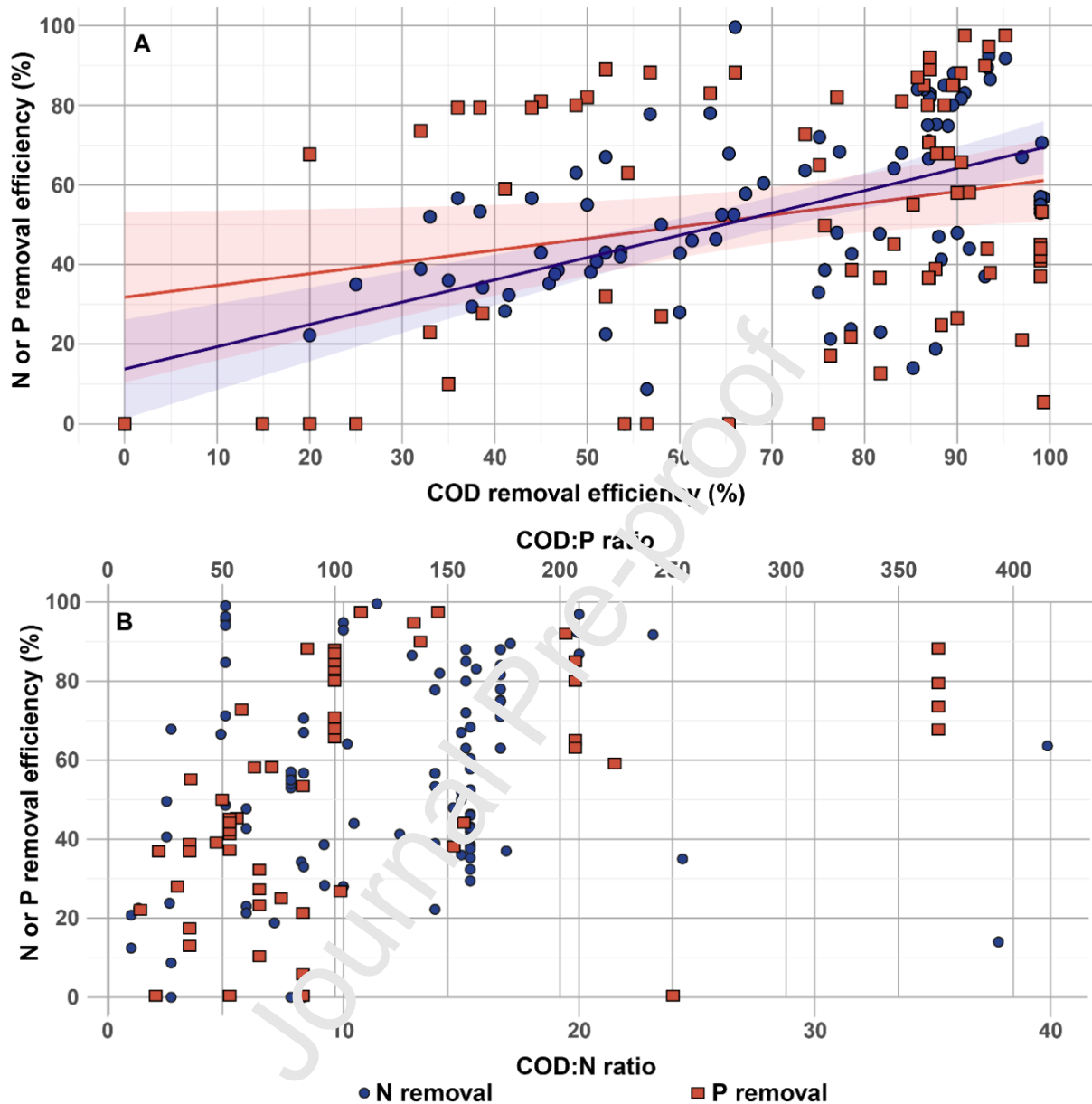**Appendix A: supplementary figures and tables**

Figure A1. Nitrogen and phosphorus removal efficiencies from wastewater at different (A) COD removal efficiencies and (B) influent COD:N and COD:P ratios (limited to values under 500 COD:P ratios to allow interpretation). A linear regression with 95% confidence interval is presented in Figure A1 A. COD stands for chemical oxygen demand.
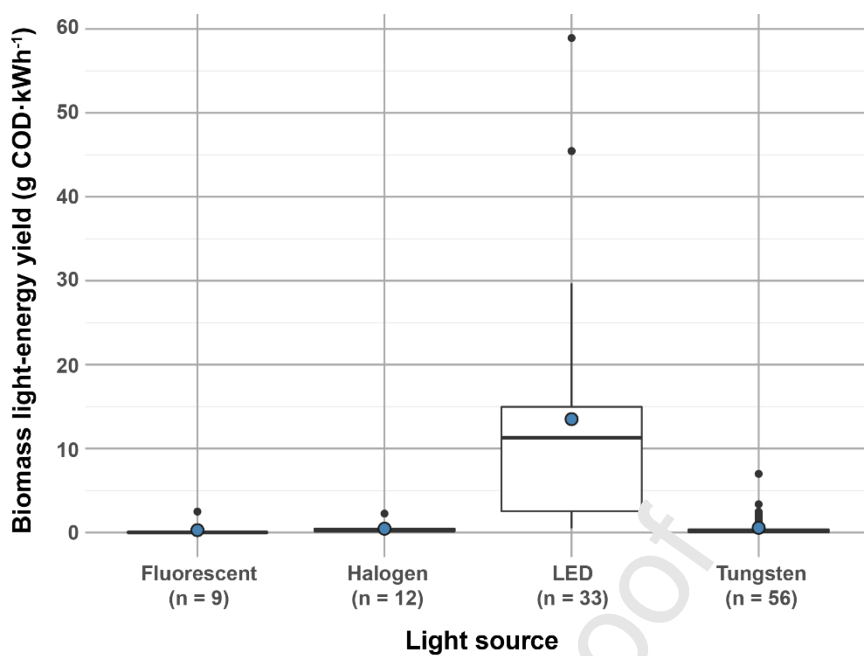
Figure A2. Photoheterotrophic biomass light-energy yields (considering only the energy consumed for illumination) according to the light source used to illuminate the reactors. The blue dots represent mean values. COD stands for chemical oxygen demand and LED for light-emitting diode.
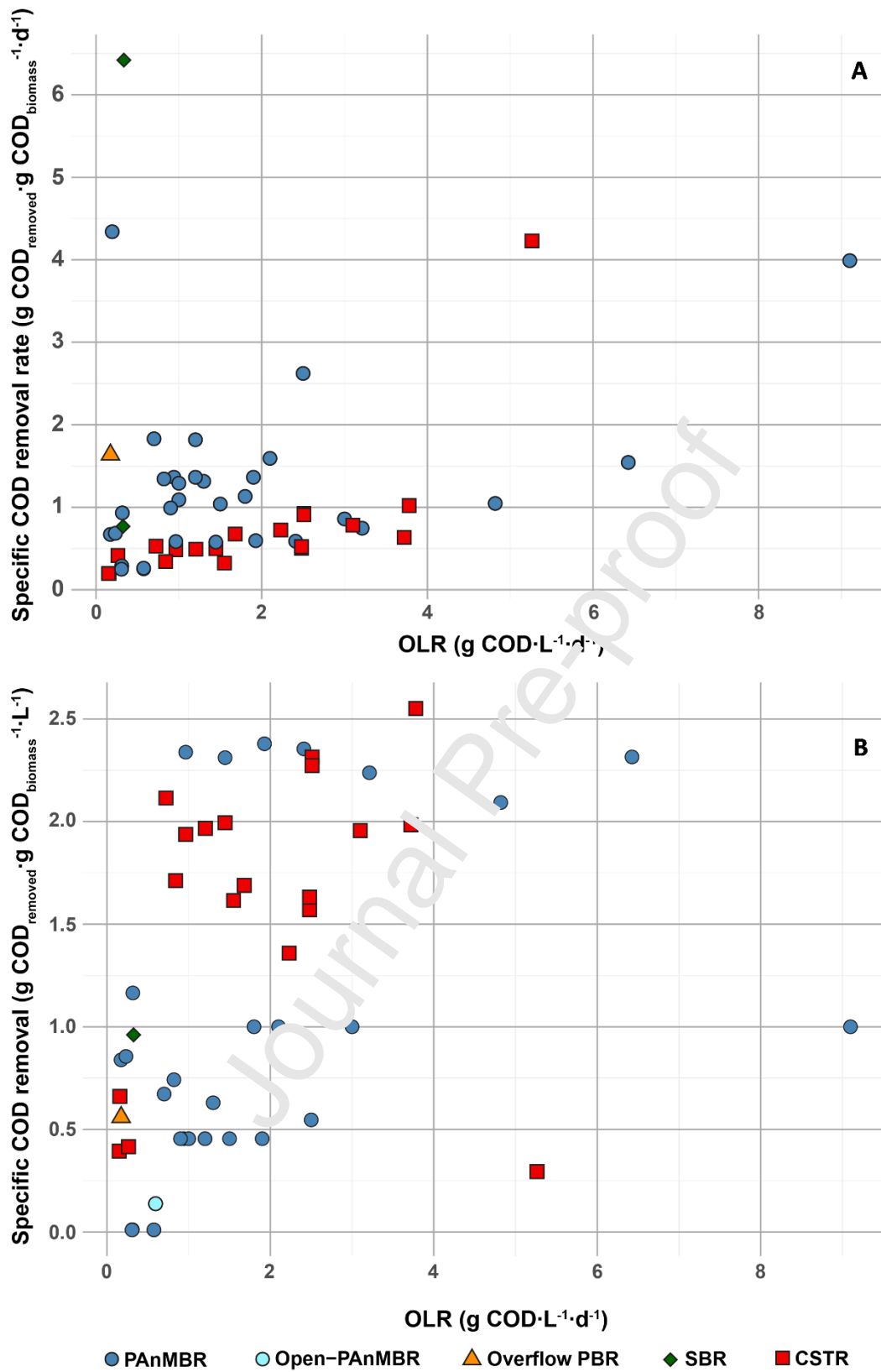
Figure A3. (A) Specific COD removal rates and (B) specific COD removal efficiencies at increasing OLRs for different reactor configurations: photo-anaerobic membrane bioreactor

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(PAnMBR; ⬤), open-PAnMBR (◉), overflow photobioreactor (PBR; △), sequencing batch reactor (SBR; ◆) and continuous stirred-tank reactor (CSTR; ■). COD stands for chemical oxygen demand and OLR for organic loading rate. To avoid overestimations, only values calculated from reasonable biomass productivities were considered.
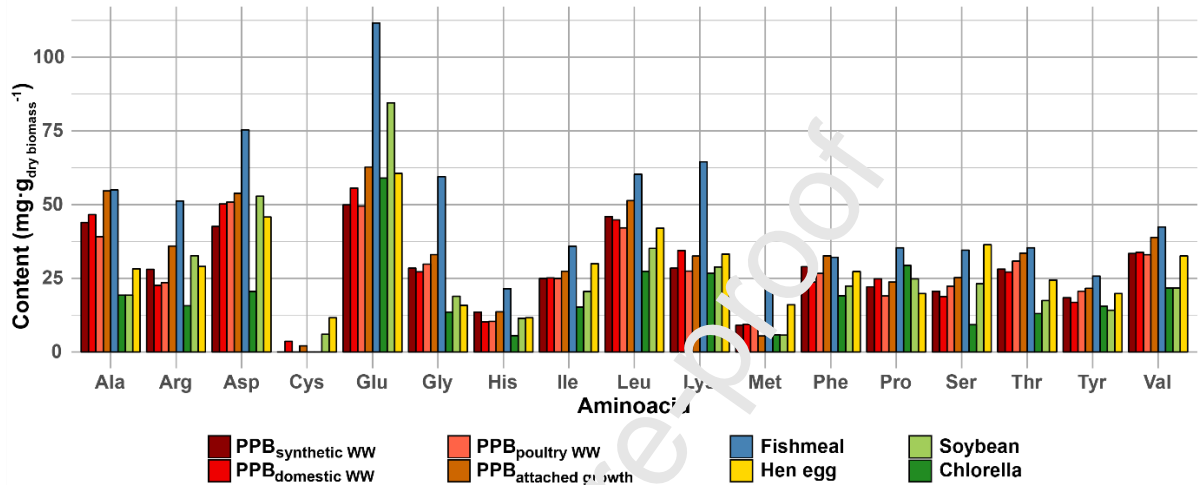

Figure A4. Amino acid profiles in non-axenic PPB biomass cultivated in different wastewaters and growth strategies. The data are compared with different common commercial feeds. Data taken from (Delamare-Debouteville et al., 2019; FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS, 1981; Hülsen et al., 2018a, 2016b). PPB stands for purple phototrophic bacteria.
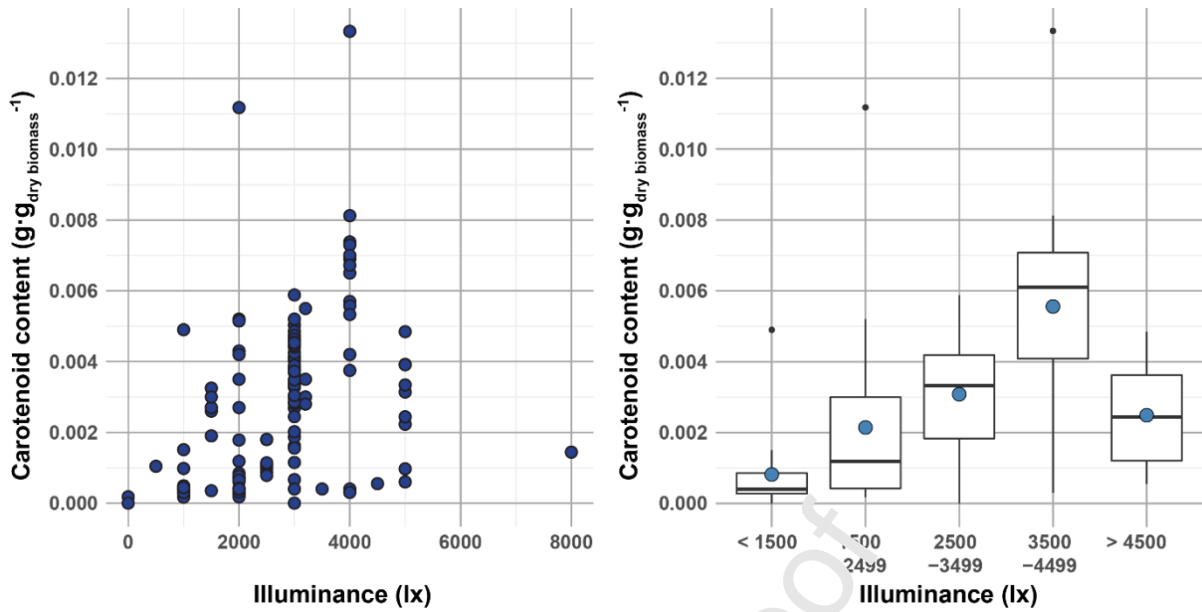
Figure A5. Carotenoid contents in the PPB biomass at different light intensities.
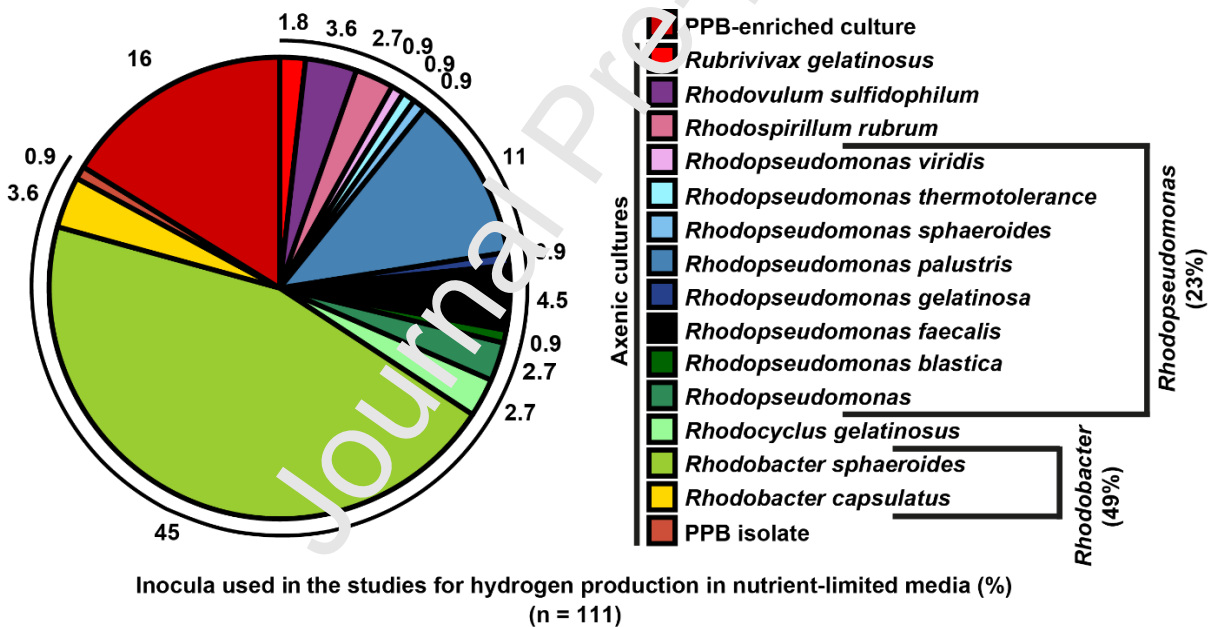

Figure A6. Inocula used for hydrogen production in nutrient-limited media using PPB. The numbers represent the corresponding percentages of a total of 71 studies. PPB stands for purple phototrophic bacteria. The data from "PPB-enriched culture" corresponds to enriched cultures grown on non-sterile, complex media.
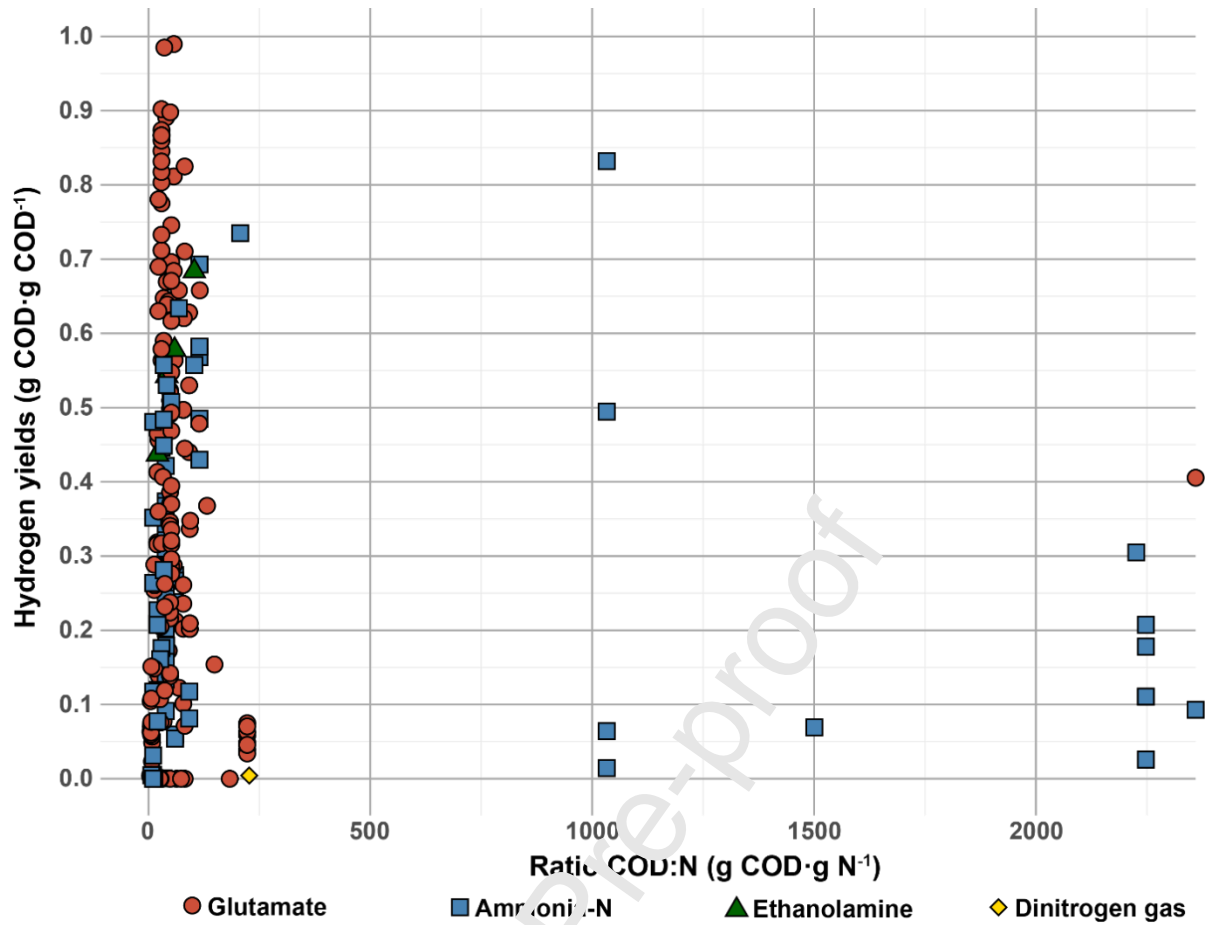
Figure A7. Hydrogen yields achieved with PF at increasing COD:N ratios in the substrates. The different compounds used as N-source for PPB growth are presented separately.
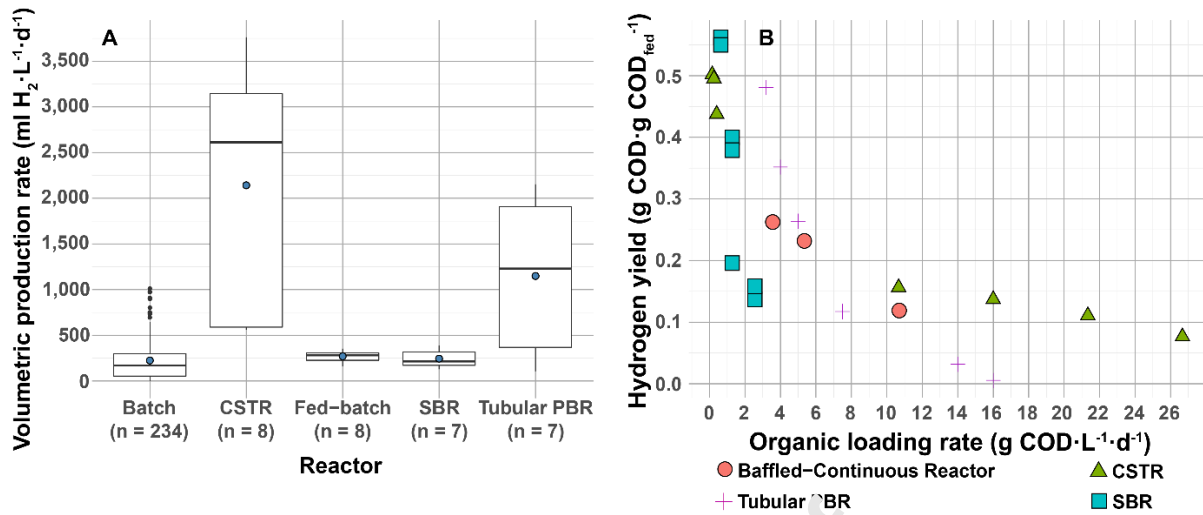
Figure A8. (A) Hydrogen production rates for different reactor configurations and (B) reported hydrogen yields at increasing organic loading rates. Only the reactor configurations with three or more independent values (n ≥ 3) are presented. COD stands for chemical oxygen demand, CSTR for continuous-stirred tank reactor, SBR for sequencing batch reactor and PBR for photobioreactor.
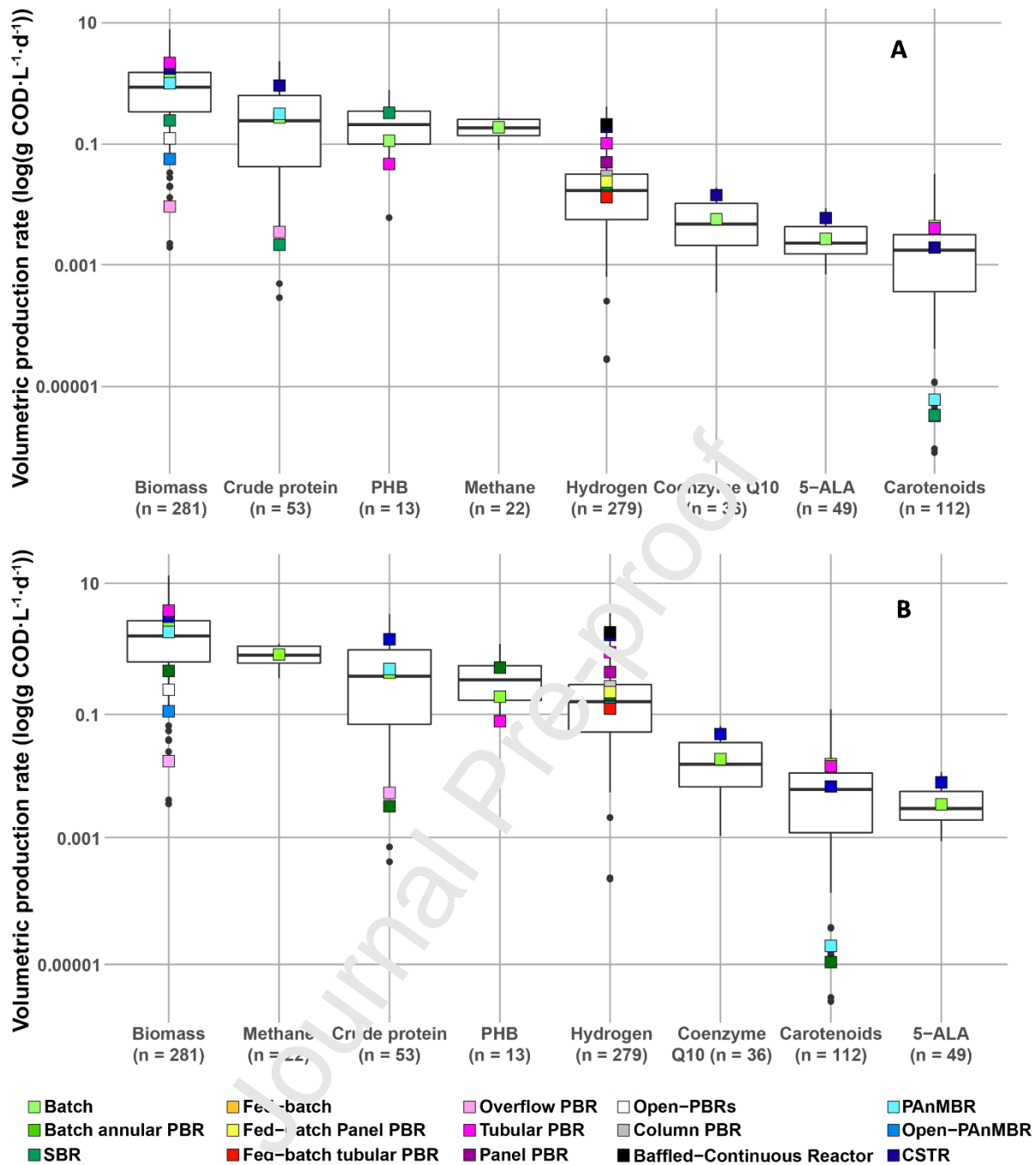
Figure A9. Volumetric production rates of each potential product in (A) mass and (B) COD units. Methane refers to the methane produced via anaerobic digestion (Hülsen et al., 2020). The coloured squares represent the mean values achieved with different reactor configurations. PHB stands for Poly(3-hydroxybutyrate), 5-ALA for 5-aminolevulinic acid, PBR for photobioreactor, PAnMBR for photo-anaerobic membrane bioreactor, SBR for sequencing batch reactor and CSTR for continuous stirred-tank reactor.

Table A1. Complete database used for the study.

Table A2. Main results regarding PHB production using PPB. PHB stands for Poly(3-hydroxybutyrate), PPB for purple phototrophic bacteria, nr for "non-reported", PBR for photobioreactor, SBR for sequencing batch reactor, VFA for volatile fatty acid and DF for dark fermentation.

Inoculum	Reactor	Substrate	PHB content ($g \cdot g_{dry\,biomass}^{-1}$)	PHB production rate ($mg \cdot L^{-1} \cdot d^{-1}$)	Reference
Rhodobacter sphaeroides	Batch	Acetic acid	0.51	nr	(Brandl et al., 1991)
Different PPB strains	Batch	Succinic + acetic acids	0-0.32	nr	(Liebergesell et al., 1991)
Rhodobacter sphaeroides	Batch	Acetic acid	0.39-0.70	nr	(Hustede et al., 1993)
Rhodobacter sphaeroides	Batch	Different simple organics	0.01-0.38	nr	(Khatipov et al., 1998)
Rhodopseudomonas palustris	Tubular PBR	Acetic acid	0.04	47	(Carlozzi and Sacchi, 2001)
Rhodopseudomonas palustris	Batch	Different simple organics	0-0.15	nr	(Mukhopadhyay et al., 2005)
Rhodobacter sphaeroides	Batch	Acetic acid	0.45	280	(Kemavongse et al., 2008)
Rhodopseudomonas palustris	Batch	Acetic acid; butyric acid	0.084; 0.053	nr	(Chen et al., 2012)
Rhodobacter sphaeroides	Batch	Acetic acid	0.51-0.54	nr	(Kim et al., 2012)
PPB-enriched culture	SBR	Acetic acid	0.20	130	(Fradinho et al., 2013a)
PPB-enriched culture	SBR	Acetic acid	0.30	210	(Fradinho et al., 2013b)
PPB-enriched culture	SBR	VFA mixture	0.50	230	(Fradinho et al., 2014)
PPB-enriched culture	SBR	Acetic acid	0.35-0.60	100-260	(Fradinho et al., 2016)
Rhodobacter sphaeroides	Batch	DF effluent	0.82	51	(Luongo et al., 2017)
PPB-enriched culture	Batch	DF effluent	0.21	6	(Luongo et al., 2017)
Rhodobacter capsulatus	Batch	VFA mixture	0.11-0.24	nr	(Montiel Corona et al., 2017)
PPB-enriched culture	SBR	Acetic acid	nr	350-770	(Fradinho et al., 2019)

Appendix B: conversion factors and assumptions considered

- For the product yields reported per biochemical oxygen demand (BOD) unit, BOD was converted to chemical oxygen demand (COD) based on the COD:BOD ratios of the feed organic material.

- Average yields reported per total chemical oxygen demand (TCOD) in the influent were recalculated using the average COD removals.

- If not specified, the terms "g dry biomass" or "g dry cells" were considered as VS.

- If not specified, the term "g dry solids" was considered as TS.

- If not reported otherwise, the VS and volatile suspended solids (VSS) contents were assumed to be equal.

- If not reported, the ratio TCOD/VS of PPB biomass was assumed to be 1.7 (Hülsen et al., 2016b).

- If not reported, the composition of the biomass was assumed to be $CH_{1.8}O_{0.38}N_{0.18}$ (Puyol et al., 2017).

- If not reported, an organic fraction of total solids (VS_{frac}) of 90% was assumed.

- If yeast extract was added to the system as nutrient supplement, its contributions to the TCOD, nitrogen and phosphorus contents were considered to be negligible (based on the relative low amounts added and its low degradability; see, for instance (Ghimire et al., 2016; Prachanurak et al., 2014; Zhang et al., 2017)).

- If not reported, a COD of 1.18 g COD·g^{-1} (that of cellulose) was assumed for lignocellulosic-rich substrates.

- If not specified, incandescent lamps were assumed to have the emission spectrum of tungsten lamps.

- If not reported, the initial biomass concentrations in batch reactors were considered to be negligible (assuming that the initial values were negligible when compared to the

final biomass concentrations; see, for instance (Alloul et al., 2019; Saejung and Thammaratana, 2016)).

- To determine potential carotenoid yields, the carotenoids concentrations in batch reactors were taken at the maximum values instead of at the end of the batch.

- For PHB calculations, a molecular weight of 22.4 g·mol C^{-1} and a COD:mass ratio of 1.44 g COD·g^{-1} were considered.

- COD:mass ratios were calculated according to Moscoviz *et al.* (2018).

- Gas volumes (including hydrogen) were normalized to standard temperature and pressure (STP) conditions (0 °C and 1 atm) and assumed as such if the conditions were not reported.

- If the hydrogen yields were given per unit of simple sugar fed, it was assumed that the simple sugars were composed of glucose (COD of 1.07 g COD·g^{-1}).

- The COD contents of sugars, protein and lipids were considered to be 1.19 g COD·g^{-1}, 1.42 g COD·g^{-1} and 2.90 g COD·g^{-1}, respectively (Moscoviz et al., 2018).

- The COD:mass ratio of carotenoids was assumed to be that of lycopene (3.22 g COD·g^{-1}).

- Hydrogen yields are given per unit of substrate COD fed to the reactors.

- Biomass yields are given per unit of substrate COD removed from the influent.

- Biomass light-energy yields (expressed in $COD_{biomass}$·kWh^{-1}) were calculated considering the biomass produced per unit of light energy supplied (*i.e.* considering the light supply as sole energy consuming process).

- Although *Rhodopseudomonas sphaeroides* has been reclassified to either *Rhodopseudomonas palustris* or *Rhodobacter sphaeroides*, the authors have decided to keep the original name presented in old studies (it was not possible to correctly attribute any of the latter species).

- When the species of bacteria was unknown, the genus was used for characterization.

- All the prices were calculated as US dollars.

- When the predominant genera/species in enriched cultures are given, they correspond to the most abundant organisms found amongst the studies that reported the structures of the microbial communities.

Appendix C: a comment on the effect of oxygen on PPB growth

A comment on the effect of oxygen on PPB growth must be made even if it is not within the exact scope of this review, as it seems unclear. On one hand, several studies have pointed out that even low dissolved oxygen (DO) levels have a negative effect on PPB phototrophic growth rates and yields, leading to loss of pigmentation and eventually to PPB wash-out (Izu et al., 2001; Jiao et al., 2003; Siefert et al., 1978; Yue et al., 2015). On the other hand, other studies have reported increased wastewater treatment efficiencies when aeration was provided (H. Lu et al., 2013; Lu et al., 2019a; Meng et al., 2018, 2017; Peng et al., 2018; P. Wu et al., 2012; Yang et al., 2018; Zhao and Zhang, 2014; Zhou et al., 2016). These latter studies suggest that micro-aerobic (0.5-1.0 mg DO·L^{-1}) and even aerobic conditions (2.0-4.0 mg DO·L^{-1}) have a positive effect of the performance of PPB-based systems (Meng et al., 2017). This lack of agreement is relevant when considering that the treatment of streams with high DO levels using phototrophic bacteria is clearly interesting (*i.e.* in in-situ aquaculture nutrient recovery). It has been previously established that PPB require an ORP below -200 mV for efficient photoheterotrophic growth (Ormerod, 1983; Siefert et al., 1978), with the negative effect of DO being explained by three main reasons: (i) the oxidation of the pigments in the PPB biomass (Pemberton et al., 1998), (ii) suppression of light harvesting complex synthesis and (iii) slower growth via respiration (Izu et al., 2001) and competition with aerobic heterotrophs (Izu et al., 2001; Siefert et al., 1978). Considering that the studies with positive

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oxygen effects imply an even broader application spectrum of PPB, this should be further investigated, using enriched cultures and non-sterile substrates. The latter is particularly relevant, as most studies claiming a positive effect of high DO levels have been carried out using axenic cultures fed with sterile media. This implies that a shift in PPB metabolism towards aerobic respiration would not have resulted in PPB being outcompeted by aerobes, which would have probably occurred under non-sterile conditions. In addition, if PPB used predominantly oxidative phosphorylation for ATP production, the COD consumption kinetics might have been faster (with faster COD removal rates), resulting also in lower biomass yields under anaerobic and micro/aerobic conditions. In fact, the obtained yields were significantly higher without oxygen (Figure C1, p value < 0.001). This further suggests the predominance of aerobic respiration when oxygen is present. The competition between PPB and aerobic heterotrophs at these conditions deserves further study.
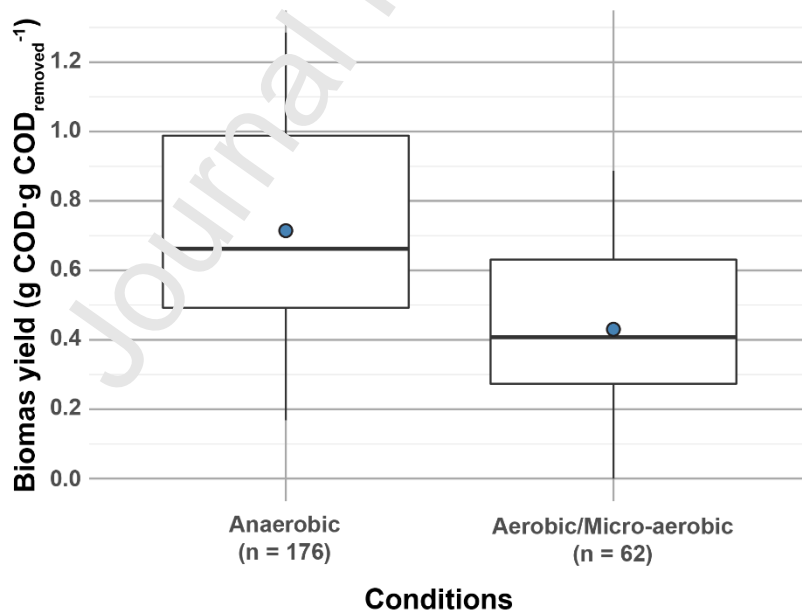

Figure C1. Biomass yields under anaerobic and aerobic/micro-aerobic conditions (dissolved oxygen between 0.5-8.0 mg·L^{-1}). The blue dots represent mean values. COD stands for chemical oxygen demand.

Appendix D: influence of operational parameters on the hydrogen yields obtained by photofermentation (PF)

The high number of studies focused on PF allows to draw generalized conclusions regarding the optimal operational parameters to maximize the hydrogen yields. The most relevant are the pH, temperature, illuminance and substrate load (Al-Mohammedawi et al., 2018; Basak and Das, 2007; Dasgupta et al., 2010; Fang et al., 2005; Ghosh et al., 2017; Hallenbeck and Liu, 2016; Phankhamla et al., 2014; Shi and Yu, 2005; Tao et al., 2008; S. C. Wu et al., 2012). The impacts of these parameters on the hydrogen yields are shown in Figure D1.
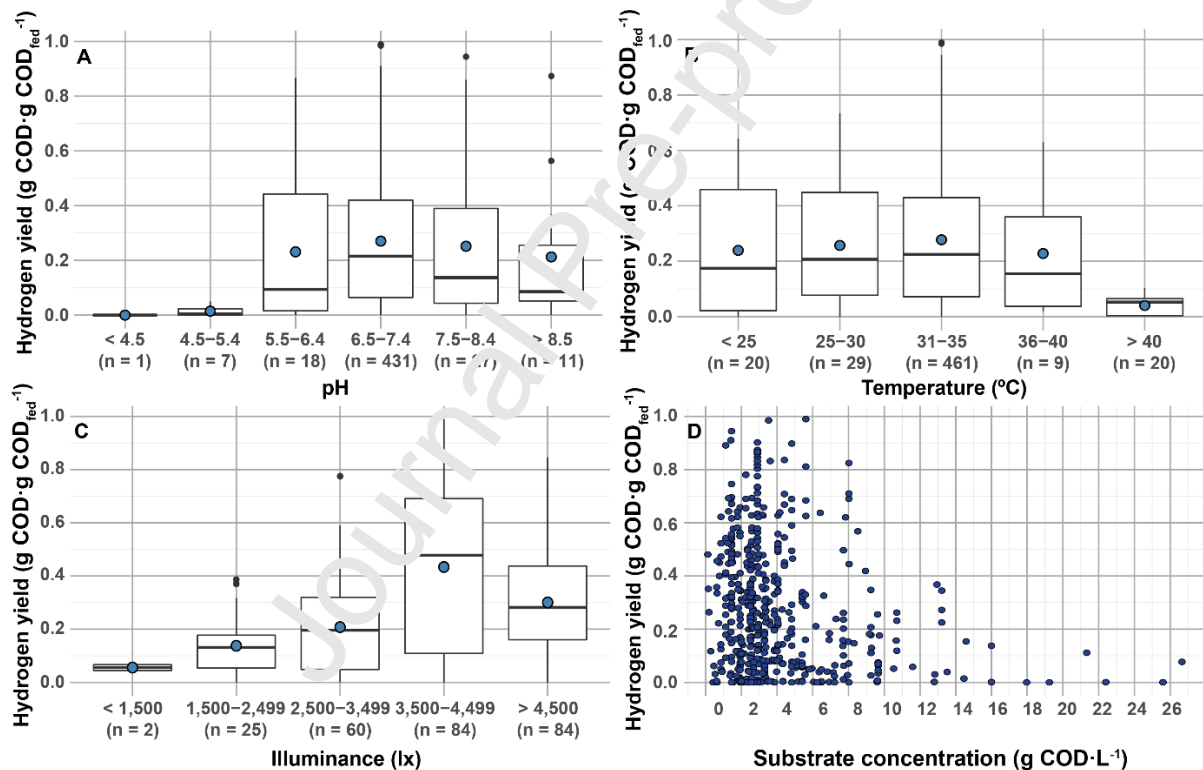

Figure D1. Hydrogen yields produced by PPB at different (A) pH values, (B) temperatures, (C) illuminances and (D) substrate concentrations. The light blue dots represent mean values. COD stands for chemical oxygen demand and PPB for purple phototrophic bacteria.

The optimal pH range for hydrogen production was found to be 6.5-7.4. Interestingly, while

low pH values (below 5.5) lead to inhibition of the hydrogen production, high pH values (over 8) did not have this effect. This suggests that PPB can thrive at high pH values. This is relevant when considering that organic acids are commonly used as substrate, implying that the substrate consumption increases the pH. The importance of pH control has been pointed out by a few authors, which concluded also that keeping the pH around 7 was relevant to maintain high hydrogen yields (Kim et al., 2011; Lazaro et al., 2015; Lee et al., 2017; Tao et al., 2008).

Regarding the temperature, most of the studies have been carried out at mesophilic temperatures (31-35 °C), which is the range showing the highest average yields. Interestingly, while values over 36-40 °C led to a clear decrease of the hydrogen yields (Figure D1B), lower temperatures did not have a significant effect on the hydrogen yields, suggesting that purple phototrophic bacteria (PPB) can thrive at low temperatures. This is in agreement with recent studies showing that PPB can efficiently grow in wastewater at temperatures of 10 °C (Hülsen et al., 2016a). This is crucial when considering that the energy requirements for reactor heating can be virtually avoided in PPB-based systems.

Continuing with the illuminance (Figure D1C), an increasing trend can be observed up to values of 3,500-4,500 lx, after which a sudden decrease in the hydrogen yields occurs. This range was also found as optimum for maximizing the carotenoids content in PPB (Section 4.3.1; Figure A5).

In agreement with the recent literature regarding biohydrogen production, Figure D1D shows another limitation when considering PF for hydrogen production (and bioprocesses for hydrogen production in general): a compromise must be found between high hydrogen yields and the treatment of substrates with high COD contents (Moscoviz et al., 2018). The hydrogen yields tend to decrease at COD concentrations over 2-4 g $COD \cdot L^{-1}$, which imposes the need to dilute the substrates in many cases, further limiting the application of PF for

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treating high strength waste streams.

Other than the COD concentration, the COD content of the substrate itself might also affect the obtained hydrogen yields. As explained previously, CO_2 will be either released and fixed depending on the reduction state of the organic compound used as carbon source. Therefore, if CO_2 is produced is significant amounts, its further fixation via the CBB cycle can be used as dissipation pathway, competing with H_2 production. Further research would be needed to elucidate the precise relationships between anabolism and hydrogen yields in PPB.

Table 1. Main results regarding coenzyme Q10 production using PPB. CSTR stands for continuous stirred tank reactor, nr stands "non-reported", and PPB for purple phototrophic bacteria.

Inoculum	Reactor	Substrate	Coenzyme Q10 content ($mg \cdot g_{dry\ biomass}^{-1}$)	Coenzyme Q10 production rate ($mg \cdot L^{-1} \cdot d^{-1}$)	Reference
Rhodobacter capsulatus	Batch	Acetic acid	2.5-4.6	3.56-5.18	(Urakami and Yoshida, 1993)
Rhodobacter sphaeroides	CSTR	Malic acid + casamino acid	4.1-6.3	10.7-19.1	(Kien et al., 2010)
Rhodobacter sphaeroides	Fed-batch	Malic acid + casamino acid	5.5-8.1	nr	(Kien et al., 2010)
Rhodobacter sphaeroides	Batch	Malic acid + casamino acid	2.0-4.7	6.12-13.7	(Kien et al., 2010)
Rhodobacter sphaeroides	Batch	Acetic acid	0.8-2.5	3.09-17.9	(Urakami and Yoshida, 1993)
Rhodobacter sphaeroides	CSTR	Acetic acid	0.9-2.3	nr	(Urakami and Yoshida, 1993)
Rhodobacter sphaeroides	Batch	Malic acid	3.8-5.7	nr	(Zhu et al., 2017)
Rhodobacter sulfidophilus	Batch	Acetic acid	3.7-3.8	13.9-16.4	(Urakami and Yoshida, 1993)
Rhodopseudomonas palustris	Batch	Acetic acid	0.2-1.5	0.36-2.41	(Urakami and Yoshida, 1993)
Rhodospirillum rubrum	Batch	Simple organics	2.3-3.3	0.78-1.29	(Tian et al., 2010a)
Rhodospirillum rubrum	Batch	Malic acid + leaf hydrolysates	2.3-9.3	2.20-4.58	(Tian et al., 2010b)

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Table 2. Current market prices and volumes of the different potential products. The potential revenue due to the content of each product within the PPB biomass is also presented. PPB stands for purple phototrophic bacteria, SCP for single-cell protein, 5-ALA for 5-Aminolevulinic acid and PHA for polyhydroxyalkanoate.

Product	Market price ($\cdot kg^{-1}$) [a]	Global market volume (kt$\cdot$year^{-1})	Compound annual growth rate (%) [b]	Average content in PPB biomass (g$\cdot$g$_{dry\,biomass}^{-1}$)	Average PPB value ($\cdot kg^{-1}$)	Reference
biomass fertilizer	0.42-0.47 [c]	119,000,000 N/ 46,000,000 P	1.4	-	0.45	(FOOD AND AGRICULTURE ORGANIZAT THE UNITED NATIONS, 2017; Schnitke TechNavio, 2018)
biomass fertilizer	0.8	-	-	-	0.80	(El-Haggar, 2007)
fishmeal	1.48-1.61 [d]	~ 80,000	11	0.51	1.14 [e]	(Reuters, 2018a; The World Bank Group,
try meal	0.60-0.80 [f]	~ 8,600	3.9	0.51	0.53 [e]	(The World Bank Group, 2019)
ean meal	0.36-0.46 [d]	~ 357,000	5.0	0.51	0.47 [e]	(The World Bank Group, 2019)
enoids	400-7,000	1,400	5.6	0.0025	9.25	(BCC Research, 2015)
ne Q10	562	1,168	9.0	0.0038	2.14	(Grand View Research; Market research and 2015)
LA	1,339	64	4.7	-	3.35 [g]	(360 Research Reports, 2019; Research Cosm
$_4$ [h]	0.54	2,569,280 [i]	9.0	0.21	0.11	(MarketWatch, 2019; Paturska et al., 2015; 2018)
$_2$	1.33-4.42	60,000	15	-	-	(Moscoviz et al., 2018; Reuters, 2018
HA	1.95-4.42	17	11	0.18	0.57	(Moscoviz et al., 2018; Reportlinker, 2

a. Excluding transportation costs

b. Predicted in 2017-2019

c. Price of diammonium phosphate (expected for 2022)

d. Varying price from January 2018 until January 2019

e. The prices were corrected to account for the protein content in fishmeal (69% (Delamare-Deboutteville et al., 2019)), poultry feed meal (67% (Delamare-Deboutteville et al., 2019)) and soybean meal (44% (Dersjant-Li, 2002))

f. Data from personal communication with protein producers

g. Calculated using the ratio PPB/5-ALA from the average productivities shown in Figure S9 (1.24 g$_{biomass}\cdot$L$^{-1}\cdot$d^{-1} and 0.0031 g$_{5-ALA}\cdot$L$^{-1}\cdot$d^{-1})

h. Considered as unconventional substitute of natural gas

i. Assuming normal conditions and common natural gas composition (96.5% CH$_4$, 1.8% C$_2$H$_6$, 0.45 C$_3$H$_8$, 0.1% iso-C$_4$H$_{10}$, 0.1% n-C$_4$H$_{10}$, 0.05% iso-C$_5$H$_{12}$, 0.03% n-C$_5$H$_{12}$, 0.07% n-C$_6$H$_{14}$, 0.3% N$_2$ and 0.6% CO$_2$) (ISO, 2006)

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Highlights

- PPB-enriched cultures and real waste matrices should be used in future studies

- Treatment efficiencies and product value are main economic drivers

- Natural illumination is required for large-scale economic feasibility

- Biomass utilization as protein-rich feed appears to be most promising