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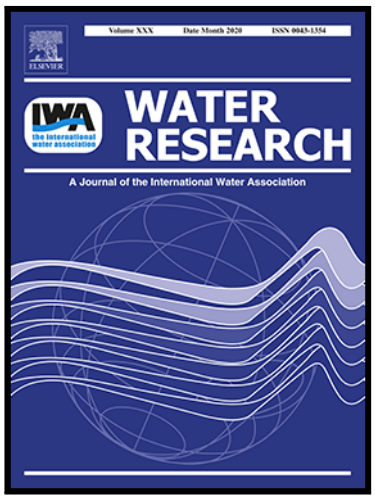

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Highlights

- Photoheterotrophic growth was suppressed in the presence of oxygen
- PPB were outcompeted by aerobic heterotrophs under aerobic conditions
- The out-competition is explained by lower aerobic growth rates of PPB
- PPB are resilient to oxygen due to their chemoheterotrophic capabilities

Purple phototrophic bacteria are outcompeted by aerobic heterotrophs in the presence of oxygen

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Abstract

There is an ongoing debate around the effect of microaerobic/aerobic conditions on the wastewater treatment performance and stability of enriched purple phototrophic bacteria (PPB) cultures. It is well known that oxygen-induced oxidative conditions inhibit the synthesis of light harvesting complexes, required for photoheterotrophy. However, in applied research, several publications have reported efficient wastewater treatment at high dissolved oxygen (DO) levels. This study evaluated the impact of different DO concentrations (0-0.25 mg·L^{-1}, 0-0.5 mg·L^{-1} and 0-4.5 mg·L^{-1}) on the COD, nitrogen and phosphorus removal performances, the biomass yields, and the final microbial communities of PPB-enriched cultures, treating real wastewaters (domestic and poultry processing wastewater). The results show that the presence of oxygen suppressed photoheterotrophic growth, which led to a complete pigment and colour loss in a matter of 20-30 h after starting the batch. Under aerobic conditions, chemoheterotrophy was the dominant catabolic pathway, with wastewater treatment performances similar to those achieved in common aerobic reactors, rather than those corresponding to phototrophic systems (*i.e.* considerable total COD decrease (45-57% aerobically *vs.* ±10% anaerobically). This includes faster consumption of COD and nutrients, lower nutrient removal efficiencies (50-58% *vs.* 72-99% for NH_4^+-N), lower COD:N:P substrate ratios (100:4.5-5.0:0.4-0.8 *vs.* 100:6.7-12:0.9-1.2), and lower apparent biomass yields (0.15-0.31 *vs.* 0.8-1.2 g $COD_{biomass}$·g $COD_{removed}^{-1}$)). The suppression of photoheterotrophy inevitably resulted in a reduction of the relative PPB abundances in all the aerated tests (below 20% at the end of the tests), as PPB lost their main competitive advantage against competing aerobic heterotrophic microbes. This was explained by the lower aerobic PPB growth rates (2.4 d^{-1} at 35 ºC) when compared to common growth rates for aerobic heterotrophs (6.0 d^{-1} at 20 ºC). Therefore, PPB effectively outcompete other microbes under illuminated-anaerobic conditions, but not under aerobic or even micro-aerobic conditions, as shown by continuously aerated tests controlled at undetectable DO levels. While their aerobic heterotrophic capabilities provide some resilience, at non-sterile conditions PPB cannot dominate when growing chemoheterotrophically, and will be outcompeted.

Keywords

Nutrient recovery; Enriched cultures; Photoheterotrophy; Wastewater; Purple non-sulfur bacteria; Purple sulfur bacteria

1. Introduction

Purple phototrophic bacteria (PPB) have been proposed as an interesting mediator to transform wastewater constituents into potentially valuable products, such as, hydrogen, polyhydroxyalkanoates, carotenoids and the biomass itself (*e.g.* single-cell protein (SCP) or fertilizer) (Capson-Tojo et al., 2020; Winkler and Straka, 2019). PPB perform anoxygenic photosynthesis (as opposed to oxygenic photosynthesis) to generate energy, which enables the assimilation of pollutants from waste streams via photoheterotrophic growth (using organic compounds as carbon source), at biomass yields around 1.0 g COD·g $COD_{removed}^{-1}$ (Hülsen et al., 2018b, 2018a, 2014; Winkler and Straka, 2019). PPB can be applied for simultaneous secondary and tertiary treatment, partitioning soluble organics, nitrogen (N) and phosphorus (P) into suspended, aggregated (flocculant or granular) or attached (biofilm) biomass. A recent review of 177 studies reported median COD, N and P removal efficiencies under illuminated-anaerobic conditions (in various configurations), of 76%, 53% and 58% respectively, with optimal COD:N:P uptake ratios around 100:6-10:1-2 (Capson-Tojo et al., 2020; Lu et al., 2019b; Puyol et al., 2017). Therefore, PPB-based processes represent a promising novel alternative for resource recovery from waste streams.

During anoxygenic photosynthesis, light energy is collected via light harvesting complexes (LHC), containing various carotenoids and bacteriochlorophylls (BChl). The harvested light energy is further transformed into chemical energy in reaction centres. BChls absorb wavelengths in the near infrared (NIR) range (780-890 nm for BChl a, and 970-1040 nm for BChl b (with common maximum absorption peaks at 800 nm, 850 nm or 1010 nm), while carotenoids absorb mostly fractions of visible (VIS) light (*e.g.*, 500 nm for lycopene or 550-555 nm for spirilloxanthin) (Canniffe and Hunter, 2014; Niedzwiedzki et al., 2015; Okubo et al., 2006; Saer and Blankenship, 2017; Wang et al., 2012)). Besides VIS-light harvesting (with further transfer of excited electrons to BChls and to reaction centres for intracellular energy generation), carotenoids also fulfil photoprotective functions (Frank and Polívka, 2009; Hartigan et al., 2002; Saer and Blankenship, 2017). These carotenoids give phototrophically grown PPB cultures their very characteristic "purple" colour.

Other than phototrophic growth, PPB can also grow without light, via fermentation (anaerobic), or chemoheterotrophic respiration (aerobically, or performing denitrification) (Capson-Tojo et al., 2020). In the presence of oxygen, energy for cellular metabolism is mostly derived from aerobic respiratory chains and virtually no BChl or carotenoids are synthesized (Zeilstra-Ryalls et al., 1998). This is because the oxidative conditions resulting from the prolonged presence of oxygen inhibit the expression of most of the genes coding for the light-harvesting antenna (responsible for energy capture) and reaction centre complexes (the main components of photosystems and responsible for energy transformation via electrical charge separation) (Bauer et al., 2003; Gregor and Klug, 1999; Sganga and Bauer, 1992; Zhu et al., 1986). Using *Rhodobacter capsulatus* as model organism, it has been found that, in the presence of oxygen, a regulator known as CrtJ responds to oxidising growth conditions by oxidising a molecular HS-HS bond to an S-S bond, which stimulates the DNA binding activity of the repressor (Bauer et al., 2003). This process is responsible for the repression of

photosynthesis gene expression, with a redox pair potential of -270 mV (Madigan et al., 2011). It has been hypothesized that repressing gene expression is a protection mechanism of the cells to avoid photooxidative damage (Zhu et al., 1986). This suppression results in the inhibition of photoheterotrophy and the shift towards chemoheterotrophy, which results in reduced biomass yields and COD:N:P substrate ratios (Izu et al., 2001; Jiao et al., 2003; Siefert et al., 1978; Yue et al., 2015). Both quantitative studies and theoretical analyses have confirmed this phenomenon under micro-aerobic conditions, with oxidative phosphorylation, rather than cyclic photophosphorylation, being the predominant ATP-production route in PPB under these conditions (Lu et al., 2011a, 2011b). This is in agreement with studies showing that PPB require an oxidation reduction potential (ORP) below -200 mV for efficient photoheterotrophic growth (Ormerod, 1983; Siefert et al., 1978), which was further supported by modelling PPB addition to activated sludge reactors (Huang et al., 2001). The latter concluded that PPB do not synthesize pigments under aerobic conditions (Huang et al., 2001). Some PPB can survive naturally in media where oxidative conditions are prevalent (such as marine sediments), but even in those situations, it is widely accepted that PPB live in niches where reducing conditions (low in oxygen) are available (Hiraishi et al., 2020; Madigan et al., 2011; Vethanayagam, 1991).

Due to the aforementioned reasons, most research applying PPB for resource recovery has been carried out under anaerobic, illuminated conditions. Nevertheless, a number of articles have suggested that photoheterotrophic growth occurred under illuminated, microaerobic (0.5-1.0 mg $DO \cdot L^{-1}$; dissolved oxygen), and even aerobic conditions (2.0-8.0 mg $DO \cdot L^{-1}$). This was suggested by either directly claiming that photophosphorylation occurred in the presence of oxygen (Meng et al., 2017; Peng et al., 2018), by reporting optimal PPB growth conditions under simultaneous light and oxygen supply (Lu et al., 2019a; Yang et al., 2018c; Zhou et al., 2016, 2015) or by reporting effective carotenoid and BChl synthesis under aerobic conditions (Lu et al., 2019a; Meng et al., 2020, 2017; Yang et al., 2018b; Zhi et al., 2020). Conversely, previous studies from the same research group reported oxidative phosphorylation as main ATP generation process in the presence of oxygen, which inherently points towards aerobic chemoheterotrophy, rather than photoheterotrophy (Lu et al., 2013, 2011a, 2011b; Wang et al., 2016). This was further supported by the generally lower biomass yields reported under aerobic conditions (0.19-0.51 g $COD \cdot g\ COD_{removed}^{-1}$ (Lu et al., 2011b; Meng et al., 2017; Wu et al., 2012)), which are far from those achieved photoheterotrophically (up to 1.0 g $COD \cdot g\ COD_{removed}^{-1}$ (Hülsen et al., 2018b, 2018a, 2014; Winkler and Straka, 2019)). Low yields point towards the consumption of COD for ATP generation (*e.g.* via oxidative phosphorylation). In addition, several studies have reported faster COD removal rates aerobically when compared to their anaerobically grown PPB (both illuminated) (Lu et al., 2019a, 2013, 2011b; Meng et al., 2020, 2017; Wang et al., 2016; Yang et al., 2018b; Zhao and Zhang, 2014). This further points towards chemoheterotrophic growth under aerobic conditions (not necessarily of PPB in mixed cultures), as growth rates for common aerobic heterotrophs are higher when compared to those of PPB growing photoheterotrophically (maximum specific growth rate for heterotrophic biomass of 6.0 d^{-1} at 20 ºC (uptake rate of 8.95 d^{-1} assuming a yield of 0.67 g $COD \cdot g\ COD^{-1}$) (Henze et al., 1987) *vs.* photoheterotrophic acetic acid specific uptake rate by PPB of 2.4 g $COD \cdot g\ COD^{-1} \cdot d^{-1}$ (20 ºC) (Puyol et al., 2017)). The aerobic uptakes rates via chemoheterotrophy for PPB have been recently determined using different pure cultures (*i.e. Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*), obtaining values of 3.2-8.0 d^{-1} at 28°C with fructose as substrate (growth rates of 1.6-4.4 d^{-1}), which are also higher than those achieved

photoheterotrophically (Alloul et al., 2021). Considering the information presented above, it is unclear how anaerobic photoheterotrophy can be performed under aerobic conditions and whether anaerobic photoheterotrophy and aerobic chemoheterotrophy take place simultaneously or are mutually exclusive.

It is also unclear how the competition of PPB with aerobic heterotrophs would play out under aerobic, non-sterile conditions, treating real wastewater. Under anaerobic, NIR-illuminated conditions, the faster growth rates compared to common anaerobic VFA-degrading microbes provide a selective advantage for PPB (particularly in the case of acetate consumption photoheterotrophically compared to methanogenesis). This is not the case under aerobic conditions. In fact, PPB have been reported to grow slower via respiration than common aerobic heterotrophs (Izu et al., 2001) and PPB are therefore likely to be outcompeted over time (Hülsen et al., 2019; Izu et al., 2001; Siefert et al., 1978). This is indicated by the very low abundances of PPB in activated sludge systems (Siefert et al., 1978; Zhang et al., 2003). It is noteworthy that most studies reporting positive effects of (high) DO levels have never considered this competition, as tests have mostly been carried out using pure/isolated cultures fed with artificial/sterile media (Liu et al., 2016, 2015; Lu et al., 2019a, 2013, 2011b; Wang et al., 2016; Wu et al., 2019, 2012; Zhou et al., 2014).

This is exacerbated by the general absence of microbial data in most studies claiming a positive effect of oxygen on the PPB treatment performance (particularly at the end in batch studies; t_{end}). Among the studies reporting the microbial communities, batch tests (with durations of 3-4 days) have shown that aerobic heterotrophs (*e.g. Pseudomonas* or *Hyphomonas*) are present at considerable proportions at t_{end} under illuminated-aerated conditions (Yang et al., 2018c, 2018a; Zhou et al., 2015), and that the PPB diversity is reduced (Zhao and Zhang, 2014). In addition, the presence of strict anaerobes at the end of batches suggests that anaerobic regions might have existed in the reactors despite aeration (Peng et al., 2018; Yang et al., 2018c). None of the aforementioned studies reported high proportions of bacteria commonly found in PPB-mediated reactors (*e.g. Rhodobacter* or *Rhodopseudomonas* (Capson-Tojo et al., 2020)), which suggests that they are not dominant or cannot compete when oxygen is supplied. Interestingly, none of these studies reported the presence of pigment-producing aerobic anoxygenic phototrophs (*i.e. Erythrobacter*, *Roseobacter*, *Roseovarius*, *Rubriomonas* or *Roseivivax*; Oren (2011)) when oxygen was provided.

In addition to obvious scientific reasons, this lack of agreement on the effect of oxygen on the performance of PPB mediated treatment systems, and its importance for resource recovery, is clearly relevant for practical application. On one hand, PPBs might be outcompeted in a matter of days. On the other, a non-detrimental effect of DO (and oxidative conditions) could expand the application spectrum of PPB (*e.g.* for in-situ aquaculture resource recovery or for recovery form high DO streams). In addition, this is highly relevant when considering the growth of PPB in open pond systems, with large surfaces for oxygen transfer, as an excessive oxygen concentration (*e.g.* due to diffusion or excessive mixing using paddle-wheels) might lead to oxidative environments if the organic matter concentration in the liquid cannot maintain reducing conditions.

This article aims to shed light on the effect of oxygen on the treatment performance of PPB-based systems, using enriched cultures and non-sterile, real wastewaters. Particular attention was paid to the competition between PPB and common aerobic heterotrophs. A number of batch tests at

different controlled DO ranges were carried out to determine the oxygen uptake rates (OURs) and the maximum DO concentrations that would allow efficient photoheterotrophic growth. A flat plate reactor was also used to study the effect of air supply at constant flow rates, to determine the lowest air flows at which photoheterotrophic growth is suppressed. The COD and nutrient recoveries, the absorption spectra (for carotenoids and BChls assessment) and the microbial populations were studied to elucidate the prevalent metabolic routes in each condition.

2. Material and methods

2.1. Wastewaters used and PPB-enriched inoculum

Two different wastewater streams, domestic and poultry-processing wastewaters, were used to further validate the applicability of the obtained results. The wastewater characteristics are presented in Table S1 in detail and briefly described here.

2.1.1. Domestic wastewater

Raw domestic wastewater was collected at a local pump station (Brisbane, Australia) and immediately stored at 4 ºC. The wastewater was settled in 200 L tanks for at least 1 day and the supernatant was used as growth media. The settled wastewater contained on average 510 ± 3 $mg \cdot L^{-1}$ total chemical oxygen demand (TCOD), 427 ± 18 $mg \cdot L^{-1}$ soluble COD (SCOD), 57 ± 5.1 $mg \cdot L^{-1}$ NH_4^+-N and 7.7 ± 0.4 $mg \cdot L^{-1}$ PO_4^{3-}-P.

2.1.2. Poultry wastewater

Wastewater from a local poultry-processing facility in Brisbane (Australia) was used. The poultry wastewater consisted of a mixture of water streams resulting from feather removal, bird degutting and general cleaning water. The raw wastewater was pre-screened on-site at 3 mm and settled. After its collection, the wastewater was stored at 4 ºC. On average, the wastewater contained 1,620 ± 299 mg $TCOD \cdot L^{-1}$, 1,386 ± 156 mg $SCOD \cdot L^{-1}$, 158 ± 11 $mg \cdot L^{-1}$ total Kjeldahl nitrogen (TKN), 37 ± 7 $mg \cdot L^{-1}$ total phosphorus (TP), 107 ± 12 NH_4^+-N and 21 ± 1.3 PO_4^{3-}-P.

2.1.3. PPB inoculum

To avoid substantial lag phases, the experiments were inoculated with a PPB-enriched sludge (50-60% PPB relative abundance). The inoculum was collected from an open outdoor demonstration-scale flat plate photobioreactor treating poultry wastewater and transferred to a 1L Schott bottle. The bottle was stirred magnetically at 150 rpm (RCT basic, Kika Labortechnik), covered with UV-VIS absorbing foil (ND 1.2 299, Transformation Tubes) and illuminated with a 150 W fluorescence lamp (Nelson Portable Flood Light) at 100 $W \cdot m^{-2}$. This procedure ensured that the inoculum would be active at the start of the experiments. The average volatile solids (VS) concentration in the inoculum (before using it) was 1.2 ± 0.4 $g \cdot L^{-1}$. It must be mentioned that the authors attempted, without success, to enrich PPB "acclimated" to microaerobic/aerobic conditions via several aerated batch experiments (see Appendix A (also see Figure A1 and A2) for the experimental setup and resulting microbial communities).

2.2. Batch experiments at different DO levels

2.2.1. Experimental setup

Based on data from the literature, four different DO ranges were tested using three open 250 mL Schott bottles as batch reactors (*i.e.* 0 mg·L^{-1}, 0-0.25 mg·L^{-1}, 0-0.5 mg·L^{-1} and 0-4.5 mg·L^{-1}), aiming at testing different DO concentrations (Meng et al., 2017; Yang et al., 2018a, 2018c). Each reactor was covered with UV-VIS absorbing foil and stirred at 150 rpm (RCT basic, Kika Labortechnik). Continuous illumination was provided by three (one per bottle) 150 W fluorescence lamps (Nelson Portable Flood Light) at an overall intensity of 120-130 W·m^{-2} (with a distribution of 17% below 700 nm, 15% at 700-800 nm, 20% at 800-900 nm and 48% at 900-1,000 nm, measured with a StellarNet BLUE-Wave Spectrometer). These lamps have been widely applied in studies growing PPB photoheterotrophically (Hülsen, 2014; Hülsen et al., 2016; Capson-Tojo et al., 2020). DO and temperature (T) were measured (M200 transmitter and Easysense O2 21 probe, Mettler-Toledo Limited, Port Melbourne, Australia) in a continuous recycle loop at a flow rate of 42-55 mL·min^{-1} (peristaltic pump, STAUFF Pumps, Richlands, Australia) and data were recorded every 15 seconds (each bottle).

The DO was kept within the desired concentrations using an on-off feedback control loop implemented in a programmable logic controller equipped with LabVIEW (LabVIEW, National Instruments, Texas, USA) (Ersahin et al., 2014). This allowed to modify the set-points as desired, simply by varying the upper DO limits (values when the air compressor would stop) and the lower DO limits (values at which the compressor would start). The air compressors were 12V DC-Permanent magnetic aerators (Hailea Ltd., China). The air flow rates into the reactors were controlled at 0.3-0.8 L·min^{-1} (unless specified otherwise) by three flow meters (BROOKS Instrument 0-1.0 L·min^{-1} air, USA). This setup allowed, not only to keep the DO within the desired limits, but also to experimentally determine the OUR. A schematic of the batch setup, including the control loop, is presented in Figure S1A.

The described setup was also used (in triplicate) as abiotic control (with acetic acid and water) to determine potential COD losses of the acetic acid due to stripping.

A similar setup, but without aeration and DO monitoring was used as anaerobic control (also run in triplicate). The bottles were closed and the headspace was extended with one empty 50 mL syringe (triplicate). The headspace of the control reactors was flushed with pure nitrogen gas.

2.2.2. Batch operation

The 0 mg·L^{-1}, 0-0.25 mg·L^{-1} and 0-0.5 mg·L^{-1} DO tests were started with 250 mL of a 1:4 vol:vol mixture of inoculum (1.2 ± 0.4 g VS·L^{-1}) and domestic wastewater. Glacial acetic acid was spiked at 500 mg COD·L^{-1} to provide a readily available source of organics and to optimise the COD:N:P ratio of the media, maximizing the nutrient removal and allowing for an easier interpretation of the results (Hülsen et al., 2016). Acetic acid was selected as substrate because: (i) it is the most common substrate used for PPB enrichment growing photoheterotrophically (Hülsen et al., 2014; Ormerod, 1983) and, (ii) it is the most common acid produced during fermentation of, non-synthetic, wastewaters (Puyol et al., 2017). The initial pH was adjusted to 6.8 ± 0.2 (with 1M NaOH).

The system was slightly modified for two particular tests. Firstly, in the experiment with DO at 0 mg·L^{-1}, the headspace of the reactors with DO control (opened to the atmosphere) was continuously flushed with nitrogen gas to ensure anaerobic conditions. Secondly, in the experiment at the highest DO level (0-4.5 mg·L^{-1}), air was not supplied by on-off control of compressors. Instead, natural air

diffusion into the liquid via droplet formation using the recycle pump was used for oxygen supply (the tube from the pump was placed slightly over the liquid level). In the same experiment, the inoculation ratio was lower than in the others (1:9 volume inoculum:volume wastewater), which caused a considerable lag phase and lower removal rates (see Figure 1-4.5 and Table 1). To avoid this issue, the inoculation ratio was increased to 1:4 in the other experiments.

Samples were taken every 5-6 h to study the kinetics of the process, measuring SCOD, TCOD, NH_4^+-N and PO_4^{3-}-P concentrations. The experiments were stopped when no further SCOD consumption was observed, leading to a duration of 31-78 h. The temperature ranged between 35 and 39 ºC.

The total solids (TS), VS, TKN and TP contents, and the absorption spectra were determined at the start and end of the experiments. The microbial communities at the start and end of the tests (for both the anaerobic controls and the aerated reactors) were studied for one flask of the triplicate tests run at 0 mg DO·L^{-1}, 0-0.5 mg DO·L^{-1}, and 0-4.5 mg DO·L^{-1}.

2.3. Estimation of the kinetic parameters of PPB respiration

The experimental setup described above was also used to evaluate the kinetics of PPB aerobic chemoheterotrophic growth (via respiration). To ensure that PPB were responsible for the aerobic uptake, a PPB culture enriched via 3 serial dilutions was used as inoculum (with an initial PPB relative abundance of 63%, Figure S2). The enrichment was carried out under anaerobic, illuminated conditions (with UV-VIS filtering foil) and using Ormerod media (as in Hülsen et al. (2014)). A Schott bottle with a working volume of 0.6 L (total volume of 1 L) was used. The cycles lasted for 6-7 days, and the serial dilutions were started with 10% of the total volume of the previous cycle. A modified Ormerod media with glucose as carbon and energy source (instead of acetic acid) was used as substrate for the aerobic test, at an initial concentration of 500 mg COD·L^{-1}. Glucose was chosen to avoid COD losses due to volatilisation and because several PPB species are known to perform glycolysis under aerobic conditions (KEGG, 2019). The test (in triplicate) was started with 250 mL of a 1:4 vol:vol mixture of inoculum (0.8 ± 0.1 g VSS·L^{-1}) and modified Ormerod medium. The DO concentration in the experiments was controlled at 1.0-3.0 mg DO·L^{-1} to ensure that aerobic conditions were maintained and that oxygen was not limiting. The temperature was 32-36 ºC. The reactors were covered with aluminium foil to avoid light penetration and phototrophic growth.

The resulting glucose consumption curve (Figure S3) was used to estimate the corresponding kinetic parameters. In agreement with most IWA mathematical models (and generally wastewater treatment modelling), a Monod kinetic function for substrate uptake was used (Batstone et al., 2002; Henze et al., 2000; Jeppsson et al., 2006; Puyol et al., 2019). The kinetic parameters (*i.e.* maximum specific uptake rate (k_{aer}; defined as growth rate (μ) divided by biomass yield (Y)) and half saturation constant ($K_{S,aer}$)) were estimated by minimization of the residual sum of squares (sum of errors of means divided by standard deviation as objective function), using de function lsqnonlin in Matlab (MATLAB R2018b, The MathWorks Inc., Natick, MA) (Batstone et al., 2002; Puyol et al., 2017). 95% confidence intervals were determined using the function nlparci. The apparent biomass yield (Y) was estimated as particulate COD increase divided by soluble COD consumption. The biomass growth rate (μ) was calculated as $\mu = k_{aer} \cdot Y$.

2.4. Flat plate batch tests at different air flow rates

2.4.1. Experimental setup

Three different conditions were tested using a flat plate reactor at increasing air flow rates (*i.e.* 0 $mL \cdot min^{-1}$, 10 $mL \cdot min^{-1}$ and 100 $mL \cdot min^{-1}$). Figure S1B shows a schematic of the flat plate reactor. The flat plate was a rectangular acrylic reactor (34 x 2 x 40 cm) with a working volume of 2.1-2.2 L and an illuminated surface/volume ratio of 30 $m^2 \cdot m^{-3}$ (Hülsen et al., 2016). Continuous illumination was provided by two 150 W fluorescence lamps (one on each side; Nelson Portable Flood Light) at an overall intensity of 120-130 $W \cdot m^{-2}$ and a wavelength spectrum similar to the one presented in Section 2.2.1. The reactor was covered with UV-VIS absorbing foil. Mixing was provided by a peristaltic pump (Cole-Parmer Masterflex L/S Pump Drive 1-100 rpm, Cole-Parmer Australia New Zealand, Chatswood, Australia), recycling reactor content from the bottom to the top of the reactor at a flow rate of 70 $mL \cdot min^{-1}$. Mixing also served for cleaning of the inner reactor wall, minimising biofilm formation. Air was supplied via a compressor (Tetra Whisper 300 Aquarium Air Pump, Tetra, Germany), connected to a flow meter (BROOKS Instrument 0-1,000 $mL \cdot min^{-1}$ air or OMEGA FLDW3209ST, OMEGA Engineering Australia, Australia) to regulate the flow rate and to an air stone (Aqua Nova Aquarium Air Stone, Geebung, Australia) placed at the bottom of the reactor. DO concentration and temperature were monitored continuously (one measurement every 30 seconds) using a Mettler-Toledo M200 transmitter coupled to an Easysense O2 21 probe (Mettler-Toledo Limited, Port Melbourne, Australia). The pH was also monitored, using a HANNAH HI8614 transmitter and a HI2910B/5 pH probe (Hanna instruments, Victoria, Australia).

2.4.2. Flat plate operation

The reactor was run in consecutive batch cycles. Before aerating the reactor, four anaerobic batches (at 0 mL $air \cdot min^{-1}$) were run to establish a baseline. The fifth cycle was aerated at 100 mL $air \cdot min^{-1}$ and the sixth cycle at 10 mL $air \cdot min^{-1}$, aiming to evaluate the effect of low and high air flow rates, and to find the lowest flow rates at which photoheterotrophic growth would be suppressed. All cycles were started with a 0.7:1.5 vol:vol mixture of inoculum and poultry wastewater. A different wastewater was used in these experiments to assess if the wastewater matrix affected the results (poultry wastewater was more complex and concentrated than domestic wastewater, see Table S1). Real wastewaters were used to evaluate microbial competition under realistic conditions (*i.e.* wastewater native competing microbes). The inoculum described in Section 2.1.3. was used for the first cycle. For the aerated tests, inoculum from the anaerobic cycles (kept in an equivalent anaerobic control) was used. The pH was not adjusted. The duration of each cycle was 3-4 days. The temperature was 35 ± 1.8 ºC.

The performance of the system was assessed by measuring the SCOD, TCOD, NH_4^+-N, PO_4^{3-}-P, TS, VS, TKN and TP contents at the beginning and end of the batch cycles. The absorption spectra and microbial communities (of the inoculum used, and of samples taken at the end of each batch run) were also analysed.

2.5. Analytical methods

TCOD and SCOD concentrations were determined using COD cell tests (Merck, 1.14541.0001, Darmstadt, Germany). The concentrations of NH_4^+-N, NO_x-N, NO_2^--N and PO_4^{3-}-P were measured via flow injection analysis (FIA; QuikChem8000, Hach Company, Loveland, USA). The concentrations of TKN and TP were determined by digestion with sulfuric acid, potassium sulphate and copper sulphate as catalysts in a block digestor (Lachat BD-46, Hach Company, Loveland, CO, USA) (Patton

and Truitt, 1992). The TS and VS contents were measured according to APHA (2005). All soluble compounds were measured after filtration through a 0.45 mm membrane filter (Millipore, Millex® -HP, Merck Group, Darmstadt, Germany). The absorption spectra were measured between 300-1,000 nm, using a spectrophotometer (Cary 50 conc, Varian).

2.6. Analysis of the microbial communities

Samples of suspended biomass were provided to the Australian Centre for Ecogenomics for DNA extraction and 16S rRNA gene amplicon sequencing using the Illumina Miseq Platform. The universal primer pair 926F (50-AAACTYAAAKGAATTGACGG-30) and the 1392wR (50-ACGGGCGGTGWGTRC-30) primer sets were used (Engelbrektson et al., 2010). Trimmomatic was used to trim the raw paired reads to remove reads shorter that 190 bp and/or with low quality (with a Phred-33 lower than 20) (Bolger et al., 2014). The trimmed paired reads were assembled using PANDAseq with default parameters (Masella et al., 2012). Removal of adapter sequences was performed using the FASTQ Clipper from the FASTX-Toolkit (Pearson et al., 1997). The resulting sequences were analysed via QIIME v1.8.0 (Caporaso et al., 2010) and de-noised with DADA2 (Callahan et al., 2016), after which the features relative frequencies of the amplicon sequence variants (ASV) were calculated. The taxonomy was assigned by uclust against the SILVA rRNA gene database (128_release) (Quast et al., 2013). ASVs with only one or two reads were filtered from the ASVs table. The resulting ASVs were processed according to Hülsen et al. (2018b).

2.7. Data treatment and statistical analyses

Statistical differences were assessed with ANOVA, carried out using the software R (The R Foundation, version 3.5.3). A 5% significance threshold was used. Oxidative COD losses were estimated calculating the area below the OUR curves (Figure S4 and Figure S5), using the trapezoidal rule in R (trapz function in the pracma package). When the OUR was not available in triplicate during a certain period, the missing values were extrapolated from the other replicates. Where confidence intervals are presented for measured data, these represent 95% confidence interval in mean from two-tailed t-test (5% significance threshold).

3. Results and discussion

3.1. Batch experiments - effect of different DO levels on photoheterotrophic growth

The COD and DO concentrations, as well as those of nutrients (*i.e.* NH_4^+-N and PO_4^{3-}-P), in the batch experiments are presented in Figure 1. The evolution of the COD and nutrient concentrations in the anaerobic controls and aerated reactors at 0 mg $DO \cdot L^{-1}$ (no aeration supplied and headspace continuously flushed with N_2) were practically the same, confirming that the unaerated setup was representative of anaerobic conditions, thus enabling unbiased comparisons of both systems (Figure 1). The absorption spectra in the anaerobic tests confirmed the presence of absorbance peaks at 450-510 nm and 800-900 nm (Figure 2), characteristic of carotenoids and BChls (Oren, 2011). The presence of LHC pigments and constant TCOD levels with biomass yields close to 1.0 g $COD \cdot g\ COD^{-1}$ evidenced the predominance of photoheterotrophic growth under anaerobic conditions (Table 1 and Figure 1A). The TCOD conservation also demonstrates biological uptake rather than losses due to stripping in the open reactors. Photoheterotrophic growth was further confirmed by the COD:N:P

removal ratios of 100:6.7-12:0.9-12, which are much higher compared to common chemoheterotrophic ratios (*e.g.* 100:5.0:0.5 for aerobic heterotrophs (Tchobanoglous et al., 2003) and well in line with previous literature values for PPB growing photoheterotrophically under anaerobic-illuminated conditions (Puyol et al., 2017).

Increasing DO levels had a substantial impact against both control and within the experiments (including the 0 mg $DO \cdot L^{-1}$ experiment). In all the aerated reactors, no characteristics pigment peaks could be detected in the absorption spectra at the end of the experiments (Figure 2), and the colour was lost (Figure S7) at all levels above 0 $mg \cdot L^{-1}$. At DO concentrations between 0-0.25 mg $DO \cdot L^{-1}$ the SCOD removal efficiency was similar between the aerated and control reactors (63% *vs.* 56%, respectively), but a considerable decrease in TCOD was observed (57% reduction *vs.* -8.4% to 3.8% in the controls; Table 1). This suggests COD consumption for ATP production rather than assimilation for growth, and a switch from anaerobic photoheterotrophy towards aerobic heterotrophic growth (via respiration). This is underlined by the substantially lower biomass yields in the aerated reactor (0.23 *vs.* 1.2 g $COD \cdot g\ COD_{removed}^{-1}$; see Table 1). Reduced biomass yields resulted in less biomass growth, which led to less N and P assimilation. This resulted in lower COD:N:P ratios of ~100:5.0:0.5 (typical for aerobic heterotrophs (Tchobanoglous et al., 2003)) and a subsequent reduction in NH_4^+-N and PO_4^{3-}-P removal efficiencies (51% aerobically *vs.* 81% anaerobically for NH_4^+-N, and 21% *vs.* 48% for PO_4^{3-}-P; see Table 1). Similar behaviours were observed in the tests at 0-0.5 mg $DO \cdot L^{-1}$ and 0-4.5 mg $DO \cdot L^{-1}$. The biomass yields were reduced to 0.15-0.31 g $COD \cdot g\ COD_{removed}^{-1}$ (compared to 0.9-1.0 g $COD \cdot g\ COD_{removed}^{-1}$ in the controls). As described above, this naturally decreased the TCOD removal efficiencies (-1.1-3.8% in the controls *vs.* 45-54% aerobically) as well as the removal of NH_4^+-N (72-82% *vs.* 50-58%) and PO_4^{3-}-P (52-44% *vs.* 37-21%) when compared to the controls, while the SCOD removal efficiencies remained similar (56-59% *vs.* 55-59%). The prevalence of biological COD removal in the aerated tests was confirmed by the abiotic control (Figure S6), were COD stripping during the first 15 h was below 40 mg $COD \cdot L^{-1}$ (period when the COD consumption in the aerated reactors occurred (see Figure 1)). This was also supported by COD balances, closing at 80-85% in the 0-0.25 and 0-0.5 mg $DO \cdot L^{-1}$ tests (the oxidized COD was estimated from the OUR (Barker and Dold, 1995)).

The removal rates of COD were also faster in aerated conditions when sufficient air was supplied (*i.e.* 0-0.5 and 0-4.5 mg $DO \cdot L^{-1}$). Aerobically, maximum values up to 92 mg $SCOD \cdot L^{-1} \cdot d^{-1}$ were achieved, higher than the maximum anaerobic rates (58 mg $SCOD \cdot L^{-1} \cdot d^{-1}$). This might be explained by the generally faster kinetics of aerobic metabolism when compared to photoheterotrophy (*e.g.* maximum specific growth rate for heterotrophic biomass of 6.0 d^{-1} (20 ºC) (Henze et al., 1987) *vs.* 2.4 d^{-1} (20 ºC) for photoheterotrophic acetic acid uptake by PPB (Puyol et al., 2017)).

To elucidate the main actors under aerobic conditions (*i.e.* PPB growing heterotrophically or common heterotrophs), the initial and final microbial communities in the batch reactors were analysed. The results show that the relative abundance of PPB was reduced when oxygen was supplied. As shown in Figure 3, the relative abundance of PPB at the end of the batches (generally dominated by *Allochromatium*, *Blastochloris* and *Xanthobacteraceae*) ranged between 36-54% in the control reactors and the experiment at 0 mg $DO \cdot L^{-1}$, while the abundance reduced to 22% in the experiment at 0-0.5 mg $DO \cdot L^{-1}$ and down to 3.3% in the experiment at 0-4.5 mg $DO \cdot L^{-1}$. Similarly, the abundances of anaerobic bacteria (mainly belonging to *Bacteroidales* or *Sphingobacteriales*) were also reduced. Instead, the relative abundances of other (non-phototrophic) aerobic organisms (*e.g.*

Pseudomonadales, *Chlamydiales* or aerobic *Eukaryota*) increased, indicating a strong competition and selective advantages for common aerobic heterotrophs under the tested conditions. The high proportions of eukaryotes (mainly aerobic protozoa such as *Intramacronucleata*) suggests that predation occurred under aerobic conditions, which explains the low biomass yields found in the aerated reactors (particularly significant in the experiment at 0-4.5 mg $DO \cdot L^{-1}$ due to the longer duration of the test). The long duration of the batch tests limited the bioavailable COD (particularly in the aerated experiments due to the faster COD uptake rates), which resulted in decay and predation by aerobic grazers. Considering that the performances of the 0-0.25 and 0-0.5 mg $DO \cdot L^{-1}$ experiments were very similar (see Figures 1 and 2), only one sample was selected for microbial analysis.

Regarding the oxidation reduction potential (ORP) in the reactors, the predominance of oxidative conditions can be confirmed by the measured DO concentrations. Within common COD ranges in activated sludge plants treating wastewater (such as those used in this study), DO concentrations of 0-1.0 mg $DO \cdot L^{-1}$ result in ORPs of 100-250 mV, and DO values over 1.0 mg $DO \cdot L^{-1}$ in ORPs of 250-370 mV, which correspond to reducing conditions with ORPs well above the point at which CrtJ is oxidised (Madigan et al., 2011; Li and Bishop, 2001). In addition, the presence of aerobic heterotrophs also evidences oxidative environments, as they require positive ORPs to grow (Jay et al., 2005). As mentioned above, these oxidative conditions (consequence of the continuous oxygen supply) are responsible for the repression of photosynthetic gene expression.

3.2. Batch experiments - evaluating the kinetics of PPB aerobic growth

A dedicated batch experiment was carried out to evaluate the kinetics of PPB growth under aerobic conditions. The resulting kinetic parameters (corresponding to a Monod kinetic function for substrate uptake; see Figure S3) were a maximum specific uptake rate (k_{aer}) of 4.66 g $COD \cdot g\ COD^{-1} \cdot d^{-1}$ and a half saturation constant ($K_{S,aer}$) of 0.4 mg $COD \cdot L^{-1}$ (at 32-36 ºC). The measured biomass yield was 0.51 g $COD \cdot g\ COD^{-1}$, resulting in a growth rate of 2.38 d^{-1}. This assumes that glucose was mostly consumed for biomass growth, as nutrients were not limiting and there was no excess of reducing power, thus avoiding the accumulation of storage compounds such as polyhydroxyalkanoates or glycogen. This value is lower than the typical growth rates reported for heterotrophic biomass (maximum specific growth rate of 6.0 d^{-1} (20 ºC) (Henze et al., 1987)), which further explains why PPB were outcompeted by aerobes. The faster removal kinetics in the aerated batch experiments (see Table 1) are in agreement with the obtained results. Recent yields and growth rates determined for pure PPB cultures (*i.e. Rhodopseudomonas palustris*, *Rhodospirillum rubrum*, *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*) on several organic substrates (*i.e.* fructose, succinate or acetic acid), also agree with the obtained results, with biomass yields of 0.50-0.76 g $COD \cdot g\ COD^{-1}$ and growth rates of 2.4-3.9 d^{-1} at 28°C (Alloul et al., 2021).

3.3. Flat plate batch tests - effect of constant air supply on photoheterotrophic growth

To determine the lowest oxygen supply that still allows photoheterotrophy and to confirm the results from the batch tests on another wastewater (and also to obtain results free of grazers), six batch cycles were carried out in a flat plate photobioreactor (Figure 4). Due to the DO sensor sensitivity of ±0.2 mg $DO \cdot L^{-1}$, we decided to control the air flow rather the the DO. The average results from Cycles 1-4 (without air supply, 0 $mL \cdot min^{-1}$) show that an anaerobic operation baseline was achieved, with removal efficiencies of 56-66%, 28-51% and 25-35% for SCOD, soluble TKN and

soluble P, respectively. As in the previous section, photoheterotrophic growth could be confirmed via TCOD conservation (balances closed to ± 10%) and by the presence of the characteristic carotenoid and BChl *a* absorption peaks at the end of the cycles (Figure 5A; note that the absorption peaks form the literature correspond to extracts, which is why the correspoding peaks in our experiments do not match perfectly).

When oxygen was supplied at an air flow rate of 100 mL·min^{-1} (Cycle 5), a similar situation to the one reported in the aerated batch reactors was observed (specifically compared to the 0-4.5 mg DO·L^{-1} test). A TCOD loss of 48% confirmed COD consumption for ATP synthesis. Respiration was confirmed by the DO measurements (Figure 5B), showing that a steep DO decrease occurred during the first 10 h of the cycle (period of the most significant COD consumption). The colour loss (Figure S8) and the absence of absorbance peaks at the end of the cycle for both carotenoids and BChl *a* (Figure 5A) also suggests that chemoheterotrophic growth (by PPB, heterotrophs, or both) was dominant. It is important to note that very small BChl peaks are still observable (Figure 5A), which might point to the presence of small amounts of pigments inside or outside the cells.

A similar behaviour was observed at an air flow rate of 10 ml·min^{-1} (Cycle 6), which resulted in non-detectable DO concentrations (≈ 0 mg·L^{-1}) during the whole experimental period, observing just a slight increase at the end. Interestingly, even at effectively 0 mg DO·L^{-1} in the reactor, pigmentation loss was still observed (no absorbance peaks, see Figure 5B; Figure S8). The loss of photoheterotrophy and the predominance of chemoheterotrophy was again underlined by TCOD losses of 44%. These results suggest that even undetectable amounts of oxygen (in several publications referred to as micro-aerobic conditions) can lead to oxidative conditions, which in turn result in inhibition of pigment synthesis and eventually in colour loss in PPB-enriched cultures. The evolution of the COD and DO concentrations, and the pH during Cycle 6 (10 ml·min^{-1}) is shown in Figure S9.

Analysis of the microbial communities confirmed the reduction in PPB abundance when oxygen is supplied (Figure 6). While the initial PPB abundance (48%, dominated by *Blastochloris* sp., *Rhodobacter* sp. and *Rhodopseudomonas* sp.) was maintained in the anaerobic baseline tests (Cycles 1-4), this was reduced to 33-34% in the aerated experiments. In contrast to the previous batch results (Figure 3), in these experiments grazers were not found and therefore the PPB decrease can simply be attributed to the growth of aerobic microbes. In fact, while the presence of aerobes initially and during the anaerobic baseline was negligible (0.2-0.4%), heterotrophs such as *Simkaniaceae*, *Planctomicrobium*, *Flavobacterium* or *Caulobacter*, accounted for 14-28% of the total 16S copies at the end of the aerated experiments. These results suggest that in long-term experiments, the slower aerobic kinetics of PPB compared to common heterotrophs will result in PPB outcompetition. Nevertheless, the colour loss cannot be explained by PPB outcompetition, as PPB were still present in considerable proportions at the end of the aerated cycles (33-34%). Therefore, suppression of pigments synthesis and pigment oxidation occurred due to the presen ce of oxygen and the resulting oxidative conditions (as previously, confirmed by the growth of aerobes), without a proportional loss of the PPB community.

We note that the slightly higher TKN and TP removal efficiencies in the aerated experiment are a consequence of an enhanced hydrolysis of the complex organic matter present in the wastewater (*e.g.* VS, particulate COD and TKN, see Table S1) under aerobic conditions. Nevertheless, the removal

ratios were still higher in the anaerobic experiment (*e.g.* COD:P removal ratios of 100:1.6-1.9 and 100:0.5-0.9 for anaerobic and aerobic conditions, respectively), confirming the results obtained in the previous batch experiments. The complex nature of the poultry wastewater is also the reason why the removal efficiencies presented in Figure 4 are expressed as TKN and TP, rather than NH_4^+-N and PO_4^{3-}-P, which are consistently mobilised (from particulate TKN and TP).

3.4. Overall impact of DO on the behavior of PPB-enriched systems

The obtained results show that photoheterotrophic growth in PPB-enriched systems is inhibited under oxidative conditions resulting from the presence of oxygen. In any of the tested conditions, oxygen supply resulted in oxidative conditions, and a consequent colour/pigments loss. This led to a metabolic switch from photoheterotrophy to aerobic chemoheterotrophy, either with or without preservation of the PPB community depending on the DO level (but always with lower PPB abundance levels after aeration). This resulted in wastewater treatment parameters (in terms of removal efficiencies, COD:N:P ratios and biomass yields) similar to those achieved in common aerobic technologies rather than anaerobic phototrophic systems. The pigmentation loss likely occurred due to repression of the genes involved in pigment production, and the consequent PPB outcompetition (Pemberton et al., 1998; Yurkov and Hughes, 2017). The results also suggest that this occurs independently from the wastewater source. We argue that even a pure PPB culture in a sterile media would behave as described above, but the colour loss would simply take more time (as indicated by the high abundances of PPB in the control reactors shown in Section 3.1). This is simply explained by the dilution of the initial pigment concentration within the cells as aerobic grow occurs (without further pigment production). PPB-enrichments cultivated anaerobically (>80% PPB relative abundance) and aerated after growth (without external carbon or nutrients supply) kept colour for 5-7 days (Figure S10). This reinforces the role of cell death/division in the loss of coloration, suggesting also that PPB can conserve pigments inside their cells under aerobic conditions for considerable periods of time, provided that carbon sources allowing aerobic growth (either of PPB or others) are not present in the media.

The loss of photoheterotrophy resulted in the loss of the main competitive advantage of PPB. Therefore, they were slowly outcompeted by aerobic organisms when oxygen was supplied, which is marked by the decreased PPB relative abundance at the end of the aerated experiments. The aerobic growth rates for PPB (estimated as 2.4 d^{-1} in our experiment at 32-36 ºC (Figure S3)) are lower than the growth rates of common aerobic chemoheterotrophs (6 d^{-1} at 20 ºC (Henze et al., 1987)), which explains the observed outcompetition. Colour loss and PPB outcompetition under aerobic conditions were previously observed in an anaerobic enriched PPB reactor, where accidental oxygen leaks in the compressor resulted in overgrow of PPB by aerobes (mainly *Thalassospira*) (Hülsen et al., 2019). Izu et al. (2001) observed a similar phenomenon and attributed the outcompetition of PPB to the lower growth kinetics of PPB compared to aerobes.

Despite their reduced competitiveness, the capability of PPB to grow aerobically via respiration gives them some resilience, even if photoheterotrophic growth is suppressed. At lower DO concentrations (0-0.5 mg $DO \cdot L^{-1}$), PPB were still present at the end of the tests, underlining a degree of resilience at these DO levels. PPB likely switched to chemoheterotrophy and this ability, even if at lower rates compared with common aerobic heterotrophs, allowed them to survive in the system. Therefore, it is logical to assume that PPB contributed actively to the aerobic COD removal in the presented experiments (although to what extent is difficult to assess quantitatively). Without competition

(especially of fast-growing aerobes), PPB might very well survive and even thrive under aerobic conditions. However, the data suggest a struggle of PPB in an aerobic system, and certainly a loss of photoheterotrophic capability due to pigment loss. Looking at the differences in rates between PPB and common aerobes, PPB might be washed-out after >3-4 serial dilutions (~7 days). This explains the low PPB abundances in conventional activated sludge (CAS) systems (Siefert et al., 1978; Zhang et al., 2003). In reality, PPB will be outcompeted in a short period of time by faster growing aerobic heterotrophs in any aerobic, non-axenic, process.

Overall, the results clearly show that oxygen (and the resulting oxidative conditions) suppressed photoheterotrophic growth of PPB, while the wastewater treatment behaviour resembled aerobic systems (*e.g.* TCOD removal efficiencies of 45-57%, apparent biomass yields of 0.15-0.31 g $COD_{biomass}$·g $COD_{removed}^{-1}$, and SCOD:N:P removal ratios of 100:4.5-5.0:0.4-0.8) rather than anaerobic phototrophic systems (*e.g.* no TCOD removal, biomass yields of 1 g $COD_{biomass}$·g $COD_{removed}^{-1}$, removal ratios of 100:6.7-12:0.9-1.2, slower removal kinetics, and higher nutrient removal efficiencies), mainly due to the establishment of typical aerobic heterotrophs, which resulted in lower abundances of PPB at the end of the tests.

These findings contradict several recent publications. Using a pure culture of *Rhodopseudomonas* and artificial wastewater, Meng et al. (2017) obtained similar biomass yields at different DO levels (up to 8 mg DO·L^{-1}), also reporting that DO levels above 1 mg DO·L^{-1} could even increase the carotenoid/BChl contents when compared to anaerobic conditions. No colour loss was observed at DO levels of 0.2-0.5 mg DO·L^{-1} during the treatment of artificial brewery wastewater with a mixture of pure PPB cultures (*Ectothiorhodospira*, *Rhodobacter sphaeroides* and *Rhodopseudomonas capsulate*), reporting average carotenoid and BChl contents of 2.53 and 10.8 mg·g VS^{-1} (Lu et al., 2019a). Pigments were also found when treating synthetic wastewater using an isolated PPB mixture (with *Ectothiorhodospira* as predominant genus) at a DO of 2-4 mg DO·L^{-1}, with contents of 3.3 and 1.4 mg·g VS^{-1} for carotenoids and BChl, respectively (Zhi et al., 2020). Yang et al. (2018a) reported 6.5 mg·g VS^{-1} of carotenoids in PSB grown under dark-aerobic conditions (no light supply and over 2 mg DO·L^{-1}).

These findings are in direct contradiction with our results. A possible reason could be that in all the studies claiming a positive high DO effect, PPB could not be outcompeted, as pure cultures and sterile media were mostly used. Nevertheless, this explanation cannot justify the high pigment contents (or even increases compared with anaerobic tests) at high DO levels (and surely under oxidative environments). As most of these experiments were carried out in short batch tests, selection of DO tolerant PPB species cannot explain this observation by itself either.

3.5. Implications for industrial application

This study shows that anaerobic conditions are required for effective PPB selection. At aerobic/micro-aerobic conditions (and the resulting oxidative environments), PPB cannot grow photoheterotrophically and are therefore outcompeted by aerobic organisms, as their main competitive advantage is lost. This limits the application of PPB-based processes for nutrient recovery to systems with low oxygen contents and reductive conditions, excluding systems where one may wish to generate PPB in a sidestream from an aerobic system (*e.g.*, sidestream generation from aquaculture), though oxygen scavengers could be added to alleviate this.

Nevertheless, the aerobic capabilities of PPB allow them to grow in the presence of oxygen (at lower rates compared to aerobic heterotrophs). Therefore, longer-term aeration is required to fully outcompete PPB. This opens the door for the application of combined systems. A combination of aerobic/anaerobic intervals could be applied in outdoor systems (illuminated by sunlight). Meng et al. (2018) reported this approach in the material and methods but the results and discussion did not present any data about the actual effects on the performance and microbial competition. Recently, Alloul et al. (2021) proposed a hybrid, 2-stage system where PPB were enriched photoheterotrophically in the 1st stage, and aerobic heterotrophs were aerobically co-cultivated with the produced PPB in the 2nd stage. Via this approach, the authors reported an improved nutritional quality of the biomass, with higher protein contents and a more attractive amino-acid profile when compared to single aerobic biomass production. In agreement with our study, they observed that the proportion of PPB decreased to 10% (PNSB) after aerobic co-cultivation with aerobic heterotrophic bacteria (from over 90% PNSB in the 1st stage). Open ponds with limited passive oxygen diffusion will also be affected by the growth of aerobes, but again the aerobic capabilities of PPB will allow them to grow and, if the concentration of organic matter is high enough to allow oxygen depletion, PPB will be able to consume the remaining COD photoheterotrophically (provided that the oxygen is supplied in amounts low enough to avoid oxidative conditions at the given COD concentrations).

We believe that a potential combined process could be based on: (i) resource recovery with high biomass yields and COD:N:P ratios being efficiently provided under anaerobic conditions during daytime (photoheterotrophy) and, (ii) limited air supply during night hours to provide aerobic wastewater treatment, but with reduced biomass yields and COD:N:P uptake ratios (due to chemoheterotrophic growth). This would likely increase the COD removal efficiencies, while reducing the N and P removal efficiencies at night. Overall, the volumetric capacity of a PPB mediated system would be increased (potentially more than doubled when compared to daytime photoheterotrophy only). This would have effects on the overall size and volume of the photobioreactor, thus reducing the capital costs, while slightly increasing the operational costs due to aeration. The effects on the microbial community might be, either substantially increased PPB proportions or PPB loss, with more non-phototrophic, facultative microbes. Higher PPB proportions might occur due to the selective growth during the day, where the abundance of PPB would be relatively high just before darkness. This means that PPB could still consume a significant fraction of the supplied oxygen (albeit at lower growth rates), while facultative organisms would consume relatively less (due to the lack of oxygen during the day), meaning that they would grow but to a lesser extent compared with PPB. Over time, this might result in PPB dominance of some specialised genera. However, this will depend entirely on the adaptation of the facultative microbes. Their slower growth during daytime and the aerobic capabilities of PPB must be able to cope with the faster growth of facultative aerobes at night. Aerobic grazers would be supressed in such a system due to intermittent low-DO conditions. Instead, we expect higher volumetric loading rates and productivities at reduced HRTs for a combined aerobic/anaerobic, day/night interval system. This provides enhanced capability against a pure solar phototrophic system, which can only be fed during the day (when solar radiation is provided).

Here we propose a solution to this major problem, but more research is required to determine whether this approach is feasible. Long-term interval studies on real wastewater (ideally outdoors) should be carried out. This should include the effects of temperature variations during day and night,

as this will substantially affect the kinetics of the different microbial clades involved, which might lead to microbial shifts.

4. Conclusions

At any of the DO concentrations (0.5-4.5 mg $DO \cdot L^{-1}$) and air flow rates tested (10 and 100 $mL \cdot min^{-1}$), oxygen supply led to inhibition of pigment production and colour loss, which suppressed the photoheterotrophic capabilities of PPB. This resulted in wastewater treatment parameters similar to those of aerobic reactors, rather than anaerobic phototrophic systems (*i.e.* considerable TCOD decrease (45-57% *vs.* ± 10%), lower COD:N:P ratios (100:4.5-5:0.4-0.8 *vs.* 100:6.7-12:0.9-1.2) and lower biomass yields (0.15-0.31 *vs.* 0.8-1.2 g $COD \cdot g\ COD_{removed}^{-1}$)). Analyses of the microbial communities showed that the proportions of PPB decreased in the aerated experiments, while the abundances of aerobic heterotrophs increased. This suggests that PPB were outcompeted in these short tests, and will eventually disappear from a real treatment setup in a matter of days (depending of the retention times). This was confirmed by the lower aerobic PPB growth rates (2.4 d^{-1} at 32-36 ºC) when compared to common growth rates for aerobic chemoheterotrophs (6.0 d^{-1} at 20 ºC).

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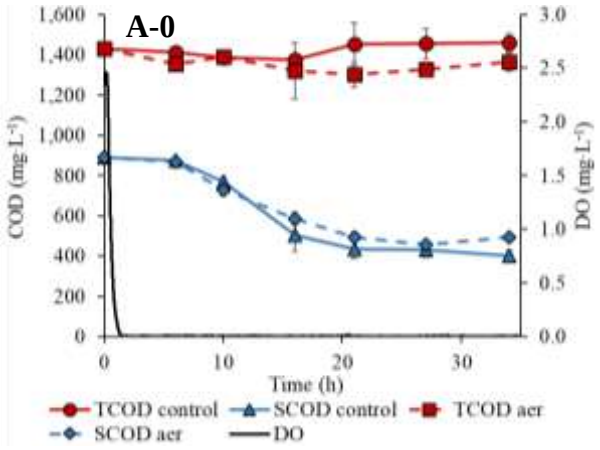
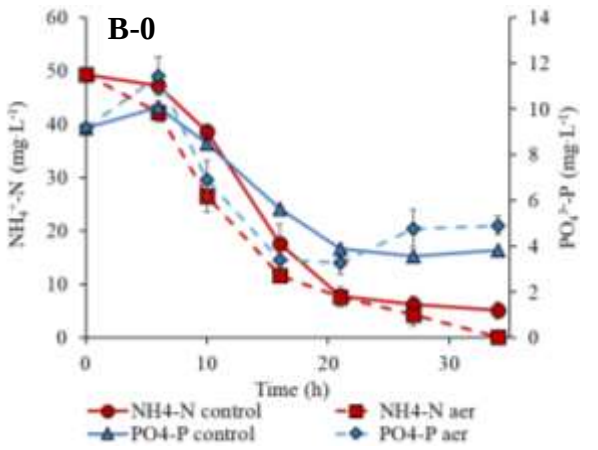
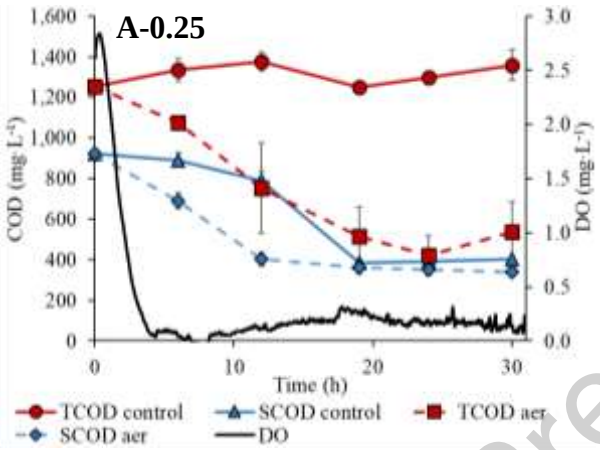
Acronyms and symbols

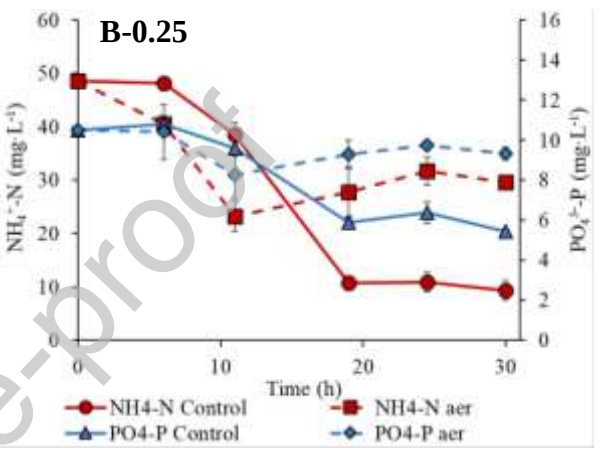
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BChl	Bacteriochlorophyll
CAS	Conventional activated sludge
COD	Chemical oxygen demand
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
FIA	Flow injection analysis
ICP-OES	Inductively coupled plasma optical emission spectrometry
IWA	International Water Association
k_{aer}	Maximum specific uptake rate
$K_{S,aer}$	half saturation constant
LHC	Light harvesting complexes
NIR	Near infrared
ORP	Oxidation reduction potential
OUR	Oxygen uptake rate
PPB	Purple phototrophic bacteria
PSB	Purple sulfur bacteria
rRNA	Ribosomal ribonucleic acid gene
SCOD	Soluble chemical oxygen demand
T	Temperature

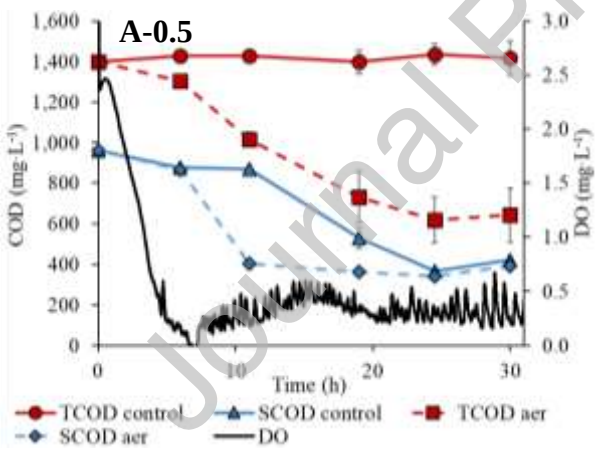
t_{end}	Time when a batch test is stopped
TCOD	Total chemical oxygen demand
TKN	Total Kjeldahl nitrogen
TP	Total phosphorus
TS	Total solids
UV-VIS	Ultraviolet-visible
VS	Volatile solids
VSS	Volatile suspended solids
Y	Biomass yield
μ	Growth rate

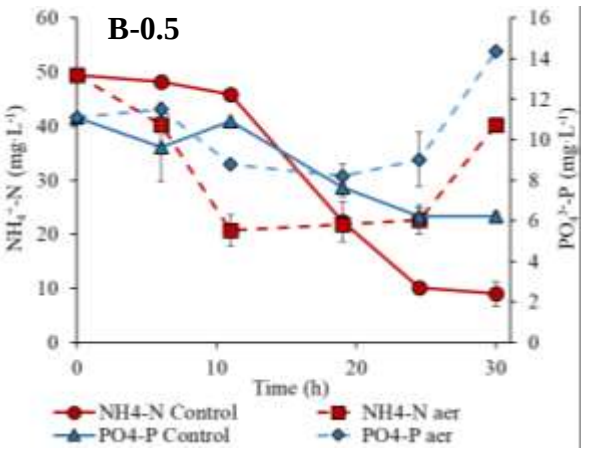


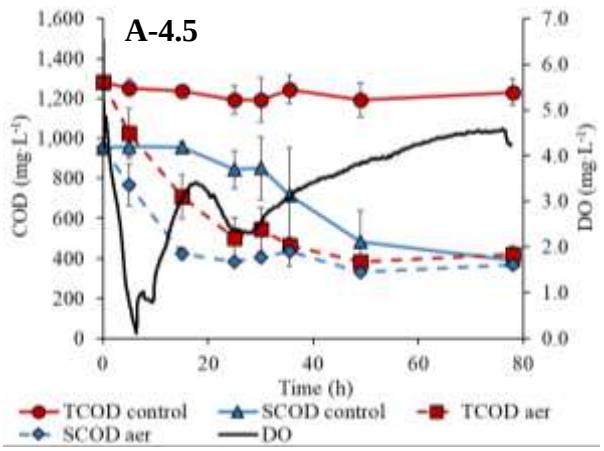












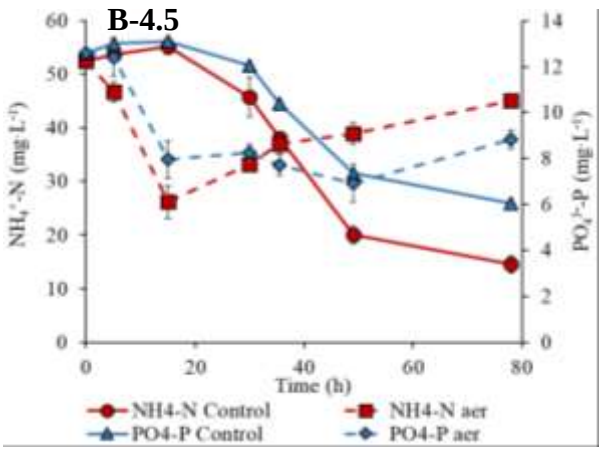


Figure 1. Evolution of (A) the TCOD, SCOD and DO concentrations and (B) the NH_4^+-N and PO_4^{3-}-P concentrations in the experiments at (0) 0 mg DO·L^{-1}, (0.25) 0-0.25 mg DO·L^{-1}, (0.5) 0-0.5 mg DO·L^{-1} and (4.5) 0-4.5 mg DO·L^{-1}. The reactors were fed with domestic wastewater spiked with acetic acid (500 mg COD·L^{-1}), illuminated at 120-130 W·m^{-2}, incubated at 35-39 ºC, and the initial pH was adjusted to 6.8 ± 0.2. The duration of test was 31-78 h. TCOD stands for total chemical oxygen demand, SCOD for soluble chemical oxygen demand and DO for dissolved oxygen. "Control" and "aer" indicate the control and aerated reactors, respectively.

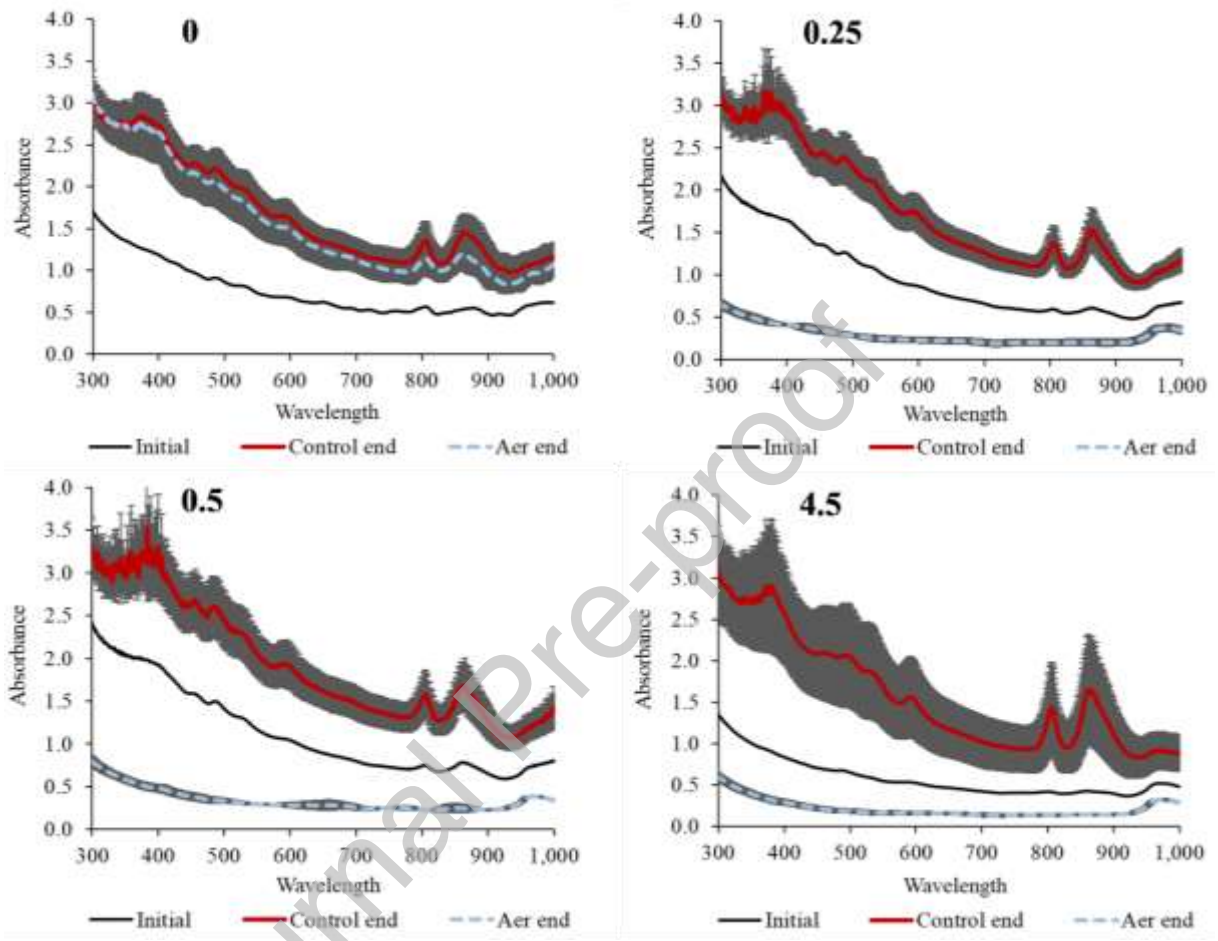


Figure 2. Initial and final absorption spectra in the experiments at (0) 0 mg DO·L^{-1}, (0.25) 0-0.25 mg DO·L^{-1}, (0.5) 0-0.5 mg DO·L^{-1} and (4.5) 0-4.5 mg DO·L^{-1}. "Control" and "aer" indicate the control and aerated reactors, respectively. Error bands represent 95% confidence interval in mean of triplicates. The reactors were fed with domestic wastewater spiked with acetic acid (500 mg COD·L^{-1}), illuminated at 120-130 W·m^{-2}, incubated at 35-39 ºC, and the initial pH was adjusted to 6.8 ± 0.2. The duration of test was 31-78 h.

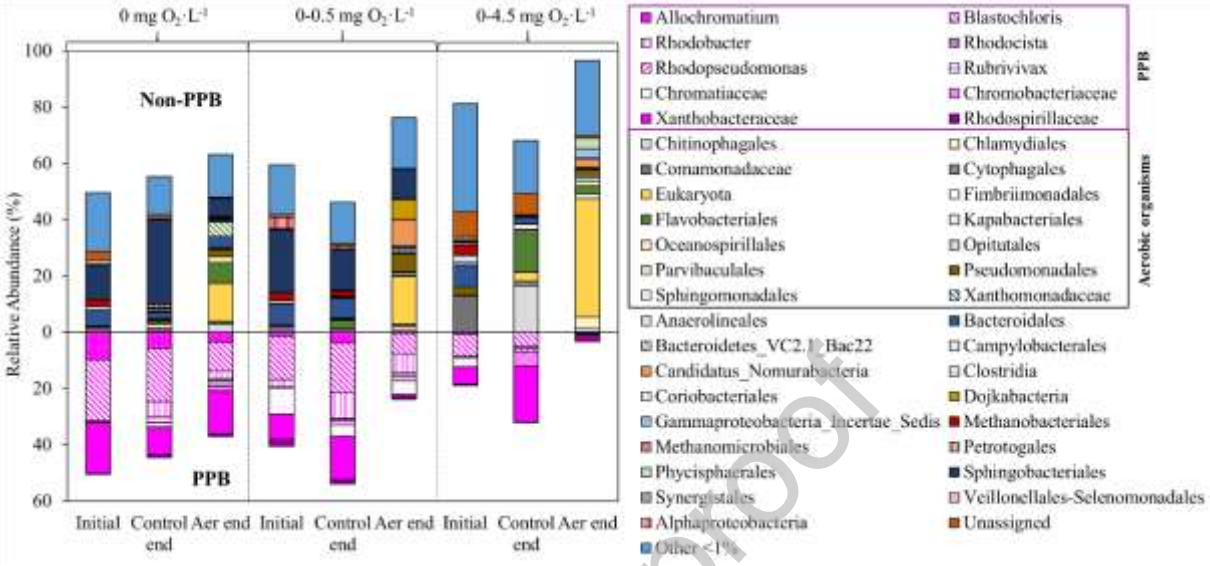


Figure 3. Structure of the microbial communities at the beginning and the end of the batch experiments. "Control" and "Aer" indicate the control and aerated reactors, respectively. The reactors were fed with domestic wastewater spiked with acetic acid (500 mg COD·L^{-1}), illuminated at 120-130 W·m^{-2}, incubated at 35-39 ºC, and the initial pH was adjusted to 6.8 ± 0.2. The duration of test was 31-78 h.

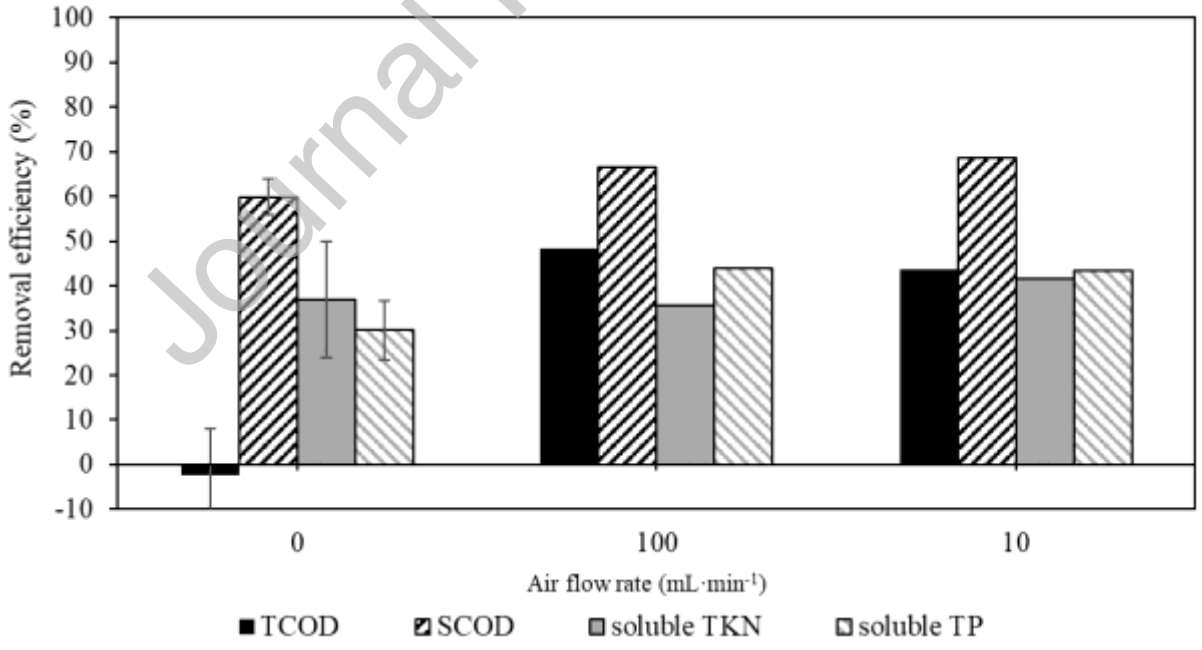


Figure 4. COD and nutrient removal efficiencies at different air flow rates in flat plate reactors. The results at 0 mL·min^{-1} correspond to the average of 4 anaerobic cycles. The reactors were fed with poultry wastewater, illuminated at 120-130 W·m^{-2}, and incubated at 35 ºC. The duration of each cycle was 3-4 d.

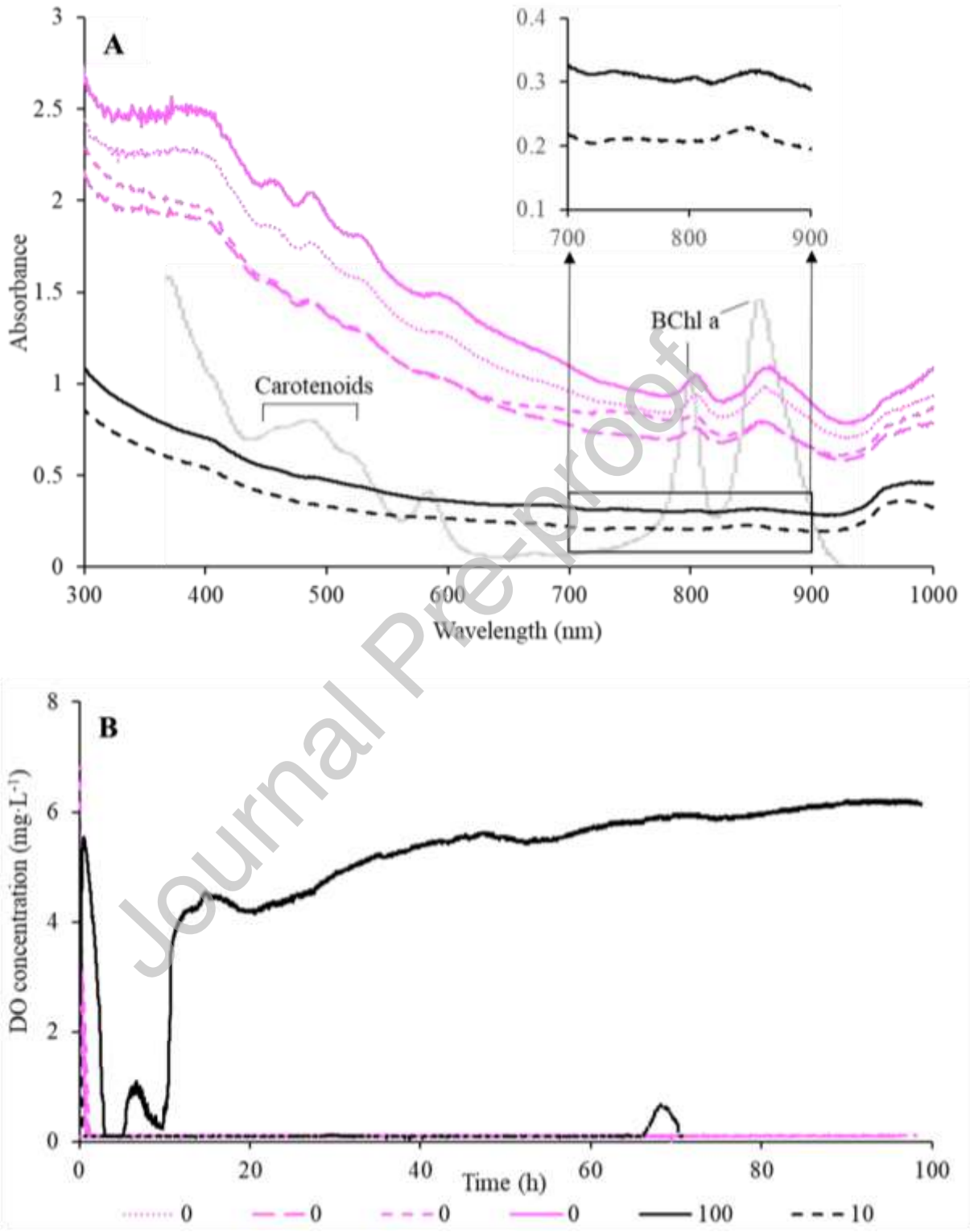


Figure 5. (A) absorption spectra and (B) dissolved oxygen (DO) concentrations at different air flow rates (presented in mL·min^{-1} under the figures). Figure A also presents a typical absorption spectrum of purple non-sulfur bacteria (grey line), consisting mainly on *Rhodobacter* and *Rhodopseudomonas* (Okubo et al., 2006; Saer and Blankenship, 2017). The reactors were fed with poultry wastewater, illuminated at 120-130 W·m^{-2}, and incubated at 35 ºC. The duration of each cycle was 3-4 d.

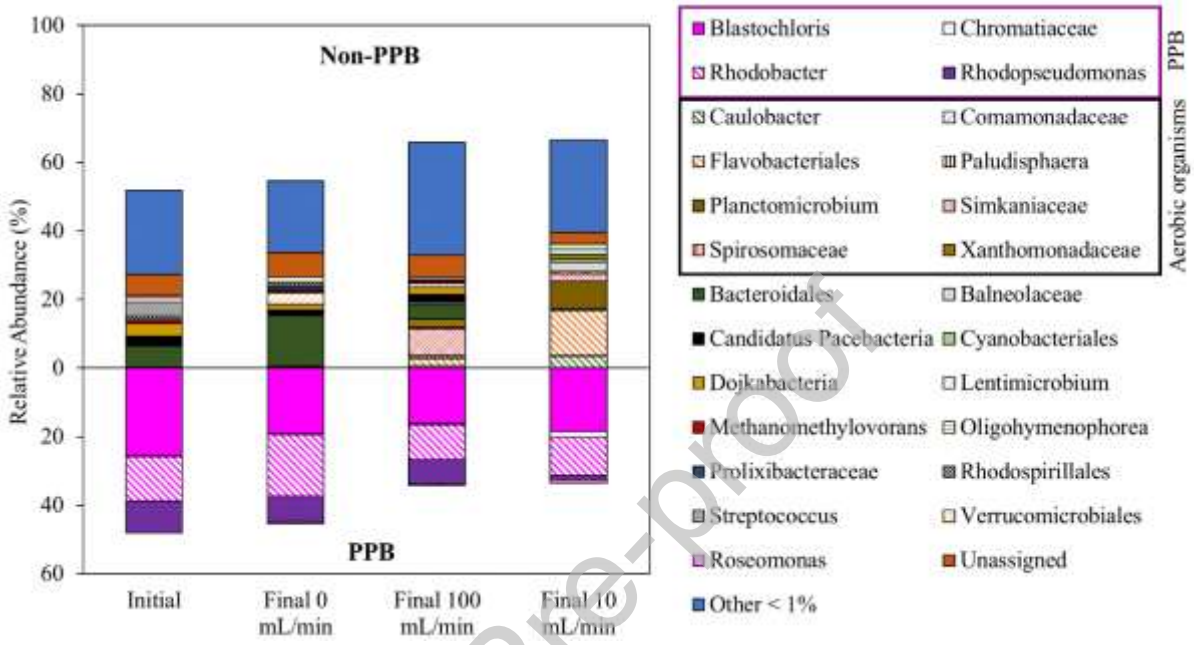


Figure 6. Structure of the microbial communities at the beginning and the end of experiments in the flat plate reactor. The reactors were fed with poultry wastewater, illuminated at 120-130 W·m^{-2}, and incubated at 35 ºC. The duration of each cycle was 3-4 d.

Table 1. Average removal efficiencies, maximum removal rates, maximum OURs, COD:N:P removal ratios and biomass yields obtained at different DO ranges. The reactors were fed with domestic wastewater spiked with acetic acid (500 mg COD·L^{-1}), illuminated at 120-130 W·m^{-2}, incubated at 35-39 ºC, and the initial pH was adjusted to 6.8 ± 0.2. The duration of test was 31-78 h. DO stands for dissolved oxygen, TCOD for total chemical oxygen demand, SCOD for chemical oxygen demand and OUR for oxygen uptake rate.

DO range in experiment (mg·L^{-1})	Condition	Removal efficiencies (%) [1]				Max. removal rate (mg·L^{-1}·h^{-1}) [1]				Max. OUR (mg·L^{-1}·h^{-1})	COD:N:P removal ratio [1]	Biomass yield (g COD·g COD^{-1}) [2]
		TCOD	SCOD	NH_4^+-N	PO_4^{3-}-P	TCOD	SCOD	NH_4^+-N	PO_4^{3-}-P			
0	Control	-2.1 ± 2.8	57 ± 0.9	90 ± 4.8	62 ± 3.1	6.5 ± 3.8	44 ± 9.4	3.5 ± 0.4	0.47 ± 0.07	n.a. [3]	100:9.0:1.1	1.0 ± 0.1
	Aerated	4.8 ± 3.1	50 ± 3.4	99 ± 0.8	66 ± 4.4	13 ± 2.2	35 ± 9.0	3.9 ± 0.4	1.1 ± 0.31	n.a. [3]	100:12:1.1	0.83 ± 0.11

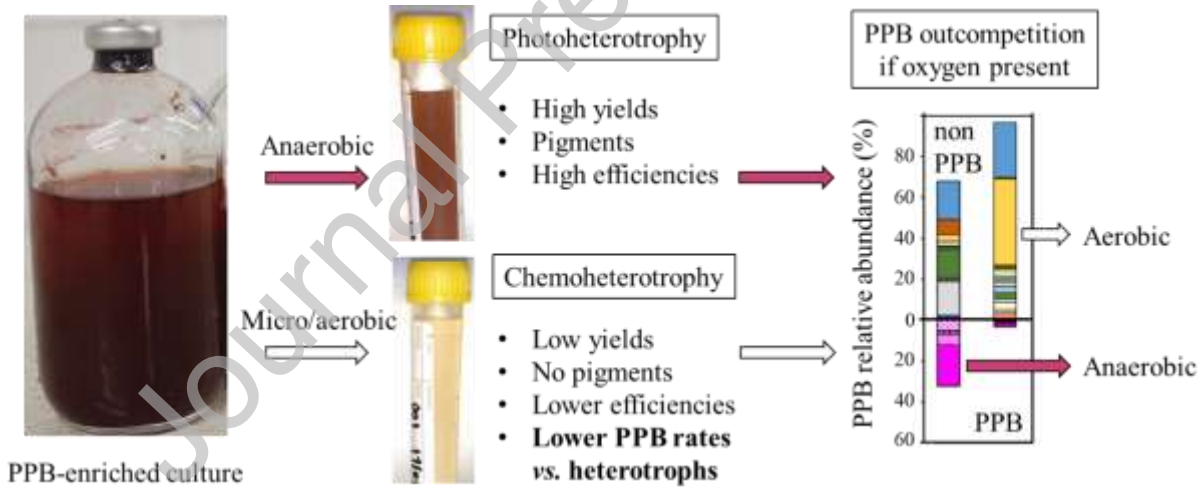
0-0.25	Control	-8.4 ± 6.1	56 ± 0.2	81 ± 3.8	48 ± 1.0	10 ± 7.7	58 ± 8.0	4.0 ± 0.5	0.53 ± 0.09	n.a. [3]	100:7.6: 1.0	1.2 ± 0.1
	Aerated	57 ± 12	63 ± 1.5	52 ± 5.9	21 ± 11	54 ± 34	48 ± 12	2.9 ± 0.7	0.35 ± 0.40	33 ± 5.3	100:4.9: 0.4	0.23 ± 0.06
0-0.5	Control	-1.1 ± 6.0	56 ± 0.3	82 ± 4.5	44 ± 1.5	3.5 ± 6.8	44 ± 5.8	2.9 ± 0.3	0.41 ± 0.05	n.a. [3]	100:7.5: 0.9	1.0 ± 0.2
	Aerated	54 ± 9.6	59 ± 1.8	58 ± 5.8	21 ± 1.4	58 ± 10	92 ± 1.9	3.9 ± 0.2	0.54 ± 0.09	34 ± 3.6	100:5.0: 0.4	0.31 ± 0.05
0-4.5	Control	3.8 ± 5.0	59 ± 3.3	72 ± 11	52 ± 4.7	5.4 ± 8.4	24 ± 15	1.4 ±1.8	0.23 ± 0.16	n.a. [3]	100:6.7: 1.2	0.9 ± 0.1
	Aerated	45 ± 8.6	55 ± 3.1	50 ± 1.4	37 ± 7.1	51 ± 25	37 ± 21	2.1 ± 0.7	0.44 ± 0.07	-	100:4.5: 0.8	0.15 ± 0.01

1. For aerated reactors, calculated before decay predominance.

2. For aerated reactors, calculated as particulate COD increase before decay divided by the SCOD decrease during that period.

3. Not applicable.

Graphical abstract



*Declaration of Interest Statement

none