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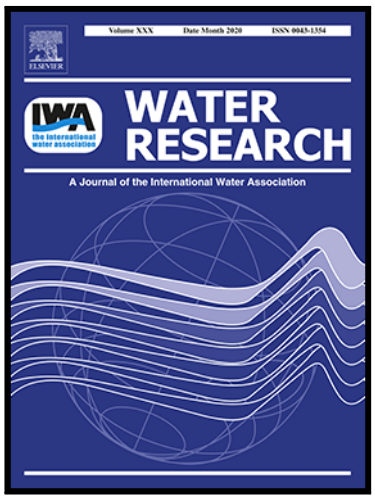


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## Highlights

- Ammonium concentrations up to 1,300 mg·$L^{-1}$ did not inhibit PPB growth
- Tolerate wide temperature range 6 °C and down to 55 °C daily range 20-30°C
- PPB biofilm harvested at ~10% DM areal production up to of 14 g TS·$m^{-2}$·$d^{-1}$
- Productivity limited by VFA rather than illumination

# Naturally illuminated photobioreactors for resource recovery from piggery and chicken-processing wastewaters utilising purple phototrophic bacteria

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## Abstract

Resource recovery from wastewater, preferably as high value products, has become an integral part of modern wastewater treatment. This work presents the potential to produce single cell protein (SCP) from pre-settled piggery wastewater (PWW) and meat chicken processing wastewater (CWW), utilising anaerobic purple phototrophic bacteria (PPB). PPB were grown as biofilm in outdoors 60 L, 80 L and 100 L flat-plate reactors, operated in sequential batch mode. PPB biofilm was recovered from reactor walls at a total solid (TS) content ~90 $g \cdot L^{-1}$, and the harvested biomass (depending on the wastewater) had a consistent quality, with high protein contents (50-65%) and low ash, potentially applicable as SCP. The COD, N and P removal efficiencies were 71±5.3%, 22±6.6%, 65±5.6% for PWW and 78±1.8%, 67±2.7% and 37±4.0% for CWW, respectively, with biofilm areal productivities up to 14 g $TS \cdot m^{-2} \cdot d^{-1}$. This was achieved at ammonium-N concentrations over 1.0 $g \cdot L^{-1}$ and temperatures up to 55 °C and down to 6 °C (daily fluctuations of 20-30 °C). The removal performances and biomass productivities were mostly dependent on the bioavailable COD in the form of volatile fatty acids (VFA). At sufficient VFA availability, the irradiance became limiting, capping biofilm formation. Harvesting of the suspended fraction resulted in increased productivities and recovery efficiencies, but lowered the product quality (*e.g.*, containing undesired inerts). The optimum between quantity and quality of product is dependent on the wastewater characteristics (i.e., organic degradable fraction) and potential pre-treatment. This study shows the potential to utilise sunlight to treat agri-industrial wastewaters while generating protein-rich PPB biomass to be used as a feed, feed additive or feed supplement.

## Keywords

purple phototrophic bacteria; wastewater; photobioreactor; resource recovery; agri-industrial

## 1. Introduction

Intensive animal farming and processing generates large volumes of wastewater with very high nitrogen, phosphorus, and organic carbon loads, requiring intensive treatment. For example, in Australia up to 550 million chicken are processed in abattoirs annually, producing on average 12L wastewater per bird (Chainetr et al. 2020). This offers a huge recovery potential of resources (e.g. around 20000, 1650, 200 tonnes of COD, TN and TP from chicken processing in Australia alone), even greater considering that there is a general trend of centralisation of animal processing activities. In addition to abattoirs (processing), intensive (centralised) animal rearing also generates large amounts of manure, which also contains substantial amounts of resources that can potentially be recovered (Orner et al. 2021, Potter et al. 2010).

The recovery of organics, nitrogen, and phosphorus from these waste streams via established technologies such as anaerobic digestion, ammonia stripping and struvite precipitation is a common practice, applied worldwide (Mehta et al. 2015). However, these technologies generate low-value products (e.g., biogas and fertilisers such as ammonium sulphate and struvite) and are hence challenged economically. In this context, the biological up-concentration of soluble organics and nutrients (e.g. nitrogen

and phosphorous) via assimilative and/or accumulative partitioning is receiving increased attention (Batstone et al. 2015).

The transformation of resources in the wastewater into single-cell protein (SCP), and/or biological fertiliser, could be a major step towards minimising resource losses in a circular economy. When grown in wastewater, microorganisms can partition up to 100% of organics and nutrients into microbial, protein-rich biomass. This is particularly applicable for phototrophic mediators, such as microalgae or purple phototrophic bacteria (PPB) (Hülsen et al. 2016b, Matassa et al. 2015, Shi et al. 2007). Phototrophic organisms can assimilate organics, ammonia and phosphate using light as energy source, rather than organics, which minimises carbon and nutrient dissipation (e.g., as CO2 or N2). When SCP is generated in a closed intensive animal production facility (e.g., pig farms) or an abattoir, it can be used to effectively recycle nutrients in the form of protein-rich biomass. This can substantially increase whole system feed conversion ratios while enhancing on-farm profitability through reduced discharge costs and the creation of additional revenue streams (e.g., fishmeal substitution in aquaculture feeds (Alloul et al. 2021b, Delamare-Debouteville et al. 2019)).

However, besides obvious regulatory, health, and safety concerns (largely depending on the wastewater source and the production process) there are several other barriers preventing widespread SCP generation from wastewater. For phototrophs, these include economic constraints, high total ammonia-N concentrations (>1.0 g·L-1) (with high pH resulting in high free ammonia concentrations (Ayre et al. 2017)), high turbidity (limiting light penetration (Barlow et al. 1975)), and long microbial adaptation times (Ayre et al. 2017). In outdoor phototrophic systems, the operational and economic

feasibility is further impacted by: (i) seasonal weather variations (e.g. dilution during rain period, evaporation during hot weather resulting in increased salinity, high or low temperatures, and illumination); (ii) by natural day/night cycles (Kumar et al. 2015); (iii) by microbial contamination (e.g. grazers); and (iv) by high biomass harvesting costs (Alabi et al. 2009, Molina Grima et al. 2003). The latter is partly caused by low biomass concentrations (e.g. 0.35 and 2 g·L-1 in open and closed photobioreactors (PBRs), respectively (Jorquera et al. 2010)). Especially in open ponds, these challenges reduce the volumetric and areal productivities (Brennan and Owende 2010, Davis et al. 2011, Jorquera et al. 2010).

Recent developments in the field of mixed culture PPB might offer solutions for some of the barriers encountered in phototrophic systems. PPB are photoheterotrophs, utilising light in the infra-red (IR) spectrum for ATP generation, and organic carbon as anabolic substrate. They offer the potential for shortened hydraulic retention times (HRT), due to faster growth rates than microalgae, and for combined secondary and tertiary treatment, due to the simultaneous removal of organics and nutrients (Hülsen et al. 2018b). The technological feasibility of this approach has been proven at laboratory scale under artificial illumination, both using batch reactors (Hülsen et al. 2018a, Marín et al. 2019), continuous PBRs (Sepúlveda-Muñoz et al. 2020) and raceways/ponds (Alloul et al. 2021a). The utilisation of native organics as carbon source avoids issues with CO2 addition, which is limiting for microalgae and is commonly added (Park and Craggs 2010). PPB have also been reported to grow at low light intensities (Dalaei et al. 2020), adapt to low temperatures (Hülsen et al. 2016a), and maintain dominance under non-axenic conditions (i.e., no sterile influent/media) (Hülsen et al. 2016a). Altogether, these

capabilities can provide some independence from varying environmental conditions (e.g., light intensity and availability, and temperature). Infra-red (IR) driven anoxygenic photosynthesis by PPB (as opposed to oxygenic photosynthesis driven by ultraviolet-visible (UV-VIS) light) under anaerobic conditions allows effective enrichment and further eliminates common aerobic grazers. The high organic content of industrial wastewater, tolerance of PPB towards ammonia (Puyol et al. 2020), and ability to use outdoor systems make PPB a good potential match for agri-industrial wastewaters.

Illumination has been previously identified as the critical cost factor, effectively prohibiting economic PPB wastewater treatment systems (Capson-Tojo et al. 2020), particularly for industrial systems (with higher organic loads). Using sunlight (filtered or natural) as energy source appears as a straight-forward solution to reduce production costs. However, outdoor studies, utilising sunlight are scarce and none of the research performed to date has dealt with real wastewater, mixed PPB cultures, or biofilms (Adessi et al. 2012, Carlozzi et al. 2010, Carlozzi et al. 2006, Carlozzi and Sacchi 2001). Consequently, the results from these studies cannot be directly extrapolated to real wastewater treatment systems, which are non-sterile and require an enriched, robust, non-axenic PPB culture, and sufficient biomass retention (Capson-Tojo et al. 2020). The use of non-sterile inputs, and the requirement to use natural light, introduce the need to consider interactions and competitions between PPB and other microbes, including other phototrophs (e.g., microalgae and cyanobacteria). Using real wastewater as substrate will also impact the harvested biomass quality and its application as feed (e.g. as supplement/additive for aquaculture), where the potential applicability of PPB

biomass as aqua feed has already been demonstrated (Banerjee et al., 2000; Chowdhury et al., 2016; Delamare-Debouteville et al., 2019; Loo et al., 2013).

To retain and concentrate biomass, photo-anaerobic membrane bioreactors have been effectively used for both microalgae and PPB-based processes (Hülsen et al., 2016; Viruela et al., 2018). However, this is expensive, and adds additional capital (e.g. 183 – 645 USD m-3 d-1) and operational costs (e.g. 0.13 – 0.27 USD m-3treated (Gao et al. 2021)). A lower cost option might be to use biofilm-based reactors, where phototrophic biomass is retained as biofilm, attached onto an illuminated surface (e.g., reactor walls). Biofilm-based systems also enable the exclusion of undesired wastewater components (e.g. inert compounds), which enhances the quality of the produced biomass (Hülsen et al. 2020). This approach can also facilitate biomass harvesting. This step, known to be particularly challenging in microalgae-based systems (accounting for over 30% of the total production costs), is often done via centrifugation, flocculation or filtration (Kadir et al., 2018). In a biofilm reactor, biomass can be directly harvested from the walls, potentially reducing costs and achieving biomass concentrations of >10% DM (Hülsen et al. 2020).

Here, we applied mixed PPB cultures in outdoor flat plate photobioreactors (FPPBRs) for growth and resource recovery from piggery (PWW) and chicken processing wastewaters (CWW), in two separate tests. The experiments using piggery wastewater were followed by those using chicken processing wastewater. Over a period of 1.5 years, the systems were run in sequential batch mode at two different locations, to assess the impacts of varying outdoor conditions (*e.g.*, day/night cycles of sunlight and temperature) on the wastewater treatment and resource recovery performance, biofilm

formation, and the microbial community. The effects of filtered (IR) *vs.* non-filtered sunlight (full spectrum) were also studied and compared. The attached and suspended biomass characteristics and its consistency were assessed in view of their potential application as SCP or fertilisers.

## 2. Materials and methods

The piggery (PWW) and chicken wastewater (CWW) tests are two different sets of experiments, separated in space and time. The PWW tests took place first, and were followed months later by the CWW tests, utilising the same equipment (with minor modifications). The PWW test served as a basis to expand and confirm the observed behaviours treating CWW, which makes the presented results complementary, rather than strictly comparable. The experiments were accompanied by several uncontrollable factors, including varying light intensities and temperatures, but also other non-weather dependant factors which complicated the comparison of data sets. The wastewaters were different. CWW contained fats, proteins, and high contents of organic matter, while PWW was almost fully degraded (in the pit), containing much less bioavailable organic matter. Nevertheless, the objective was not to test the influence of these variables over PPB growth and performance under perfectly controlled conditions, but rather to prove the technical feasibility of an outdoors, larger-scale, PPB system, able to withstand natural weather (and thus working conditions) variations.

### 2.1. Piggery wastewater (PWW)

PWW from a piggery located in Queensland (Australia) was used to feed the PBRs. The selected piggery is an intensive indoor pig farm using pressurised town water fed via valves in the bottom of the pit. The pig confinement area contains slotted floors and a shallow static pit underfoot, where the piggery waste accumulates before it is pumped into the wastewater treatment pond system. A portion of sediments is removed bi- or tri-monthly. During the operational period, the pits were completely flushed on January 9$^{th}$ (day 73, prior to batch 7) and March 16$^{th}$ of 2018 (day 139, batch 11), which drastically affected the wastewater compositions. The wastewater for this study was taken directly from the grower shed pit, pumped into a 1,000 L intermediate bulk container (IBC) and pre-settled for 30 min before being used as reactor influent. The pre-settled wastewater contained on average 4,130 (1,560) mg TCOD·L$^{-1}$ (total chemical oxygen demand), 1,590 (610) mg SCOD·L$^{-1}$ (soluble chemical oxygen demand), 1,160 (284) mg TKN·L$^{-1}$ (total Kjeldahl nitrogen), 160 (68) mg TP·L$^{-1}$ (total phosphorous), 2,420 (1340) mg TSS·L$^{-1}$ (total suspended solids), and 1,880 (325) mg VSS·L$^{-1}$ (volatile suspended solids). Numbers in brackets are standard deviations. The detailed characteristics of the wastewater can be found in supplementary materials (Table S1). Particularly after pit flushes, the VFA and SCOD concentrations in PWW were low (see Table 1). In some of these periods, glacial acetic acid was added to certain wastewater batches (as stated in Table 1) to determine the maximum removal rates and nutrient removal efficiencies without VFA limitations.

### 2.2. Meat Chicken processing wastewater (CWW)

Wastewater from a meat chicken-processing facility in Brisbane (Australia) was used. The PBRs were installed on-site, pumping the wastewater directly from its source (grit trap effluent) when needed. The raw wastewater was a mixture of water streams resulting from feather removal, bird degutting, and general cleaning water. On average, the wastewater contained 3,332 (418) mg TCOD·$L^{-1}$, 1,614 (260) mg SCOD·$L^{-1}$, 189 (30) mg TKN·$L^{-1}$, 39 (13) mg TP·$L^{-1}$, 2,713 (430) mg TS·$L^{-1}$, and 1,799 (279) mg VS·$L^{-1}$. The detailed characteristics of the wastewater can be found in supplementary materials (Table S1).

### 2.3. Purple phototrophic bacteria inoculum

No external inoculum was used at either site. PPBs were directly enriched from either pre-settled piggery wastewater or chicken processing wastewater.

### 2.4. Flat-plate photobioreactor (FPPBR) set-up

For the experiments with PWW, the set-up consisted of six custom-made acrylic FPPBR (operated in parallel), secured at the base by custom stands. Three different dimensions were used: 2 reactors with a total volume of 100 L (1x1x0.1 m), 2 of 80 L (1x1x0.08 m), and 2 of 60 L (1x1x0.06 m). Each reactor had a wall thickness of 0.015 m and had a detachable lid to cover the top. The lids were fastened via six metal clamps but were not gas tight. The illuminated reactor wall surface area was 0.85 $m^2$ per wall (1.7 $m^2$ per PBR, as some illuminated area was lost due to the reactor stands, resulting in 17, 21 and 28 illuminated $m^2 \cdot m^{-3}$ for the 100, 80, and 60 L reactors, respectively). Mixing was provided by intermittent recirculation (pulse/pause 30/30 min) of the reactor content from a port in the centre of the reactor to four ports at the reactor bottom (all with a

diameter of 1 inch). Mixing occurred only during daytime, between 05:00-18:00, working a total of 6.5 $h \cdot d^{-1}$, or 9,750 $L \cdot d^{-1}$ (~24 kWh $m^{-3}$ $d^{-1}$) (we note this constitutes excessive mixing and requires optimisation). This was carried out by the centrifuge pump P-2 (Figure 1). P-1 and P-2 were 25 $L \cdot min^{-1}$ mono pumps (0.37 kW, CP00251C1R8C, NOV Australia Pty Ltd).

The 100 L and an 80 L FPPBRs described above were later used for the tests with CWW. In these experiments, a small flow was diverted from the mixing line to the top of the reactor, where it was sprayed through a perforated tube to disperse floating fat (see dotted tube in Figure 1). A similar pumping system was used to feed the reactors with PWW. The reactors were placed in open fields available at the piggery and the chicken abattoir, and were illuminated naturally with sunlight (under natural light/dark cycles), with or without being covered with UV-VIS absorbing foil (ND 1.2 299, Lee filter, absorbing over 90% of wavelengths below 790 nm) depending on the desired illumination conditions (Table 1 and Table 2). The reactors stood vertically (without inclination), with the illuminated surfaces facing the east-west plane for both set-ups, thus maximising light availability. The dissolved oxygen (DO) concentrations, temperatures, and pH in the reactors were monitored continuously.

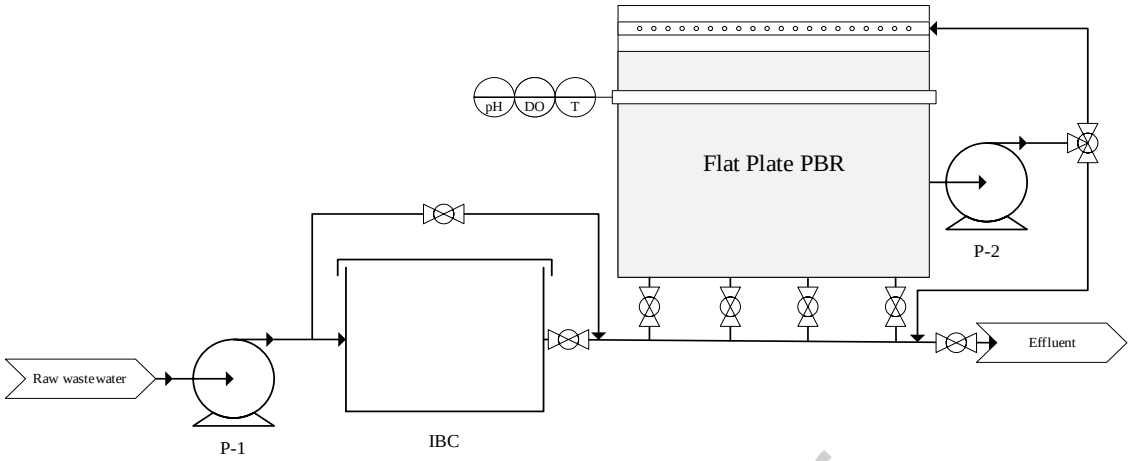


Figure 1: Schematic of the flat plate photobioreactor (FPPBR). IBC stands for intermediate bulk container, DO for dissolved oxygen and T for temperature.

### 2.5. Reactor operation

#### 2.5.1. Piggery wastewater (PWW) reactor operation

Six FPPBRs were installed and operated on-site between October 2017 and April 2018. The operation was divided into 3 phases. The controllable parameters were reactor volume, mixing, batch length and VFA availability where VFA availability (via external acetic acid addition) varied from Phase 1 to 3 and batch lengths was shortened during Phase 3. Mixing and reactor volume remained constant. The impact of these changes, which were characterised by varying batch lengths and by acetic acid addition, as well as by natural variations in the temperature and received irradiance, were assessed, to test PPB biofilm attachment, the impact of the reactor width on attachment, treatment performance, and the biofilm characteristics.

Phases 1 (Oct – Dec) and 2 (Jan – Mar) took place during spring and summer months (VFA content was varied), while Phase 3 took place during autumn months (Apr – May) (batch length and VFA was increased). This is further detailed in Table 1. The reactors

were operated in sequential batches, with batch lengths between 4.5-6.6 d, resulting in an average organic loading rate (OLR) of 0.71±0.12 g $COD \cdot L^{-1} \cdot d^{-1}$. At the start of the batch ($T_0$), each reactor was filled with PWW from the IBC (usually between 3 and 4 pm). 30% volume of reactor content from the previous batch was re-added to the tank as PPB inoculum. At the end of each batch, the reactor liquid was drained (collecting the 30% required for the next batch) and the attached biofilm was removed from the reactor walls by manual, upward scraping with a 30 cm squeegee. The collected biomass (*i.e.*, biofilm) was weighed and put on ice for transport before storage in a freezer at -4 ℃. Inoculum and fresh PWW were then re-introduced in the FPPBRs to start the next batch.

Wastewater and treated effluent were sampled from one 100, 80, and 60 L FPPBR at $T_0$ and at the end of the batch ($T_{end}$). Samples were kept on ice before being measured. Concentrations of TCOD, SCOD, $NH_4^+$-N, $NO_2^-$-N, $NO_x$-N, $PO_4^{3-}$-P, VFAs, trace elements, TKN, TP, pH, TSS and VSS were analysed. DO and temperature were continuously recorded between January and April (batches 7 – 18). The contents of TCOD, TKN, TP, trace elements, total solids (TS), and volatile solids (VS) in the harvested biomass were analysed at $T_{end}$. Amino acid and pigment contents of the wet biomass were also analysed at various time points.

Table 1. Summary of the PWW test operating conditions in the 80 L and 100 L FPPBRs.

| | **Phase 1** | **Phase 2** | **Phase 3** |
|---|---|---|---|
| | (1 – 36 d) | (40 – 70 d) | (80 – 110 d) |
| **Batch #** | 1 - 6 | 7 - 11 | 12 - 16 |
| **Season** | Spring | Summer | Autumn |
| **Batch length (d)** | 6.6 | 6.6 | 4.5 |
| **Light supply** | IR | IR | IR |
| **Acetic acid addition** | No | Yes | Yes |

| | | | |
|---|---|---|---|
| **VFA-COD at t=0* (mg·L$^{-1}$)** | 155 (117) | 476 (327) | 1,434 (715) |
| **TCOD load per batch (g TCOD·L$^{-1}$)**** | 4.03 (0.46) | 3.61 (0.46) | 3.60 (1.30) |
| **Irradiance (MJ·m$^{-2}$)***** | 23.8 (6.2) | 20.5 (7.7) | 16.9 (3.5) |

*Total VFA of PWW with Acetic acid addition; ** the average TCOD:TN ratio was 4.6±0.6; *** calculated from daily global solar exposure data from the Bureau of Meteorology weather station 040082, around 2 km away from the PWW test location (http://www.bom.gov.au), measured from midnight to midnight.

### 2.5.2. Chicken processing wastewater (CWW) reactor operation

Two FPPBRs, one of 100 L and another of 80 L, were installed and operated on-site between March 2019 and December 2019 to confirm the biofilm attachment and characteristics as well as the impact on the treatment performance. For the CWW tests, higher degradable COD concentrations were tested, and batches started with shorter batch length (as determined in the PWW tests), which changed the feeding regime. The reactors were fed twice per week, with batch cycles of 3-4 d (average cycle length of 3.5(0.6) d), resulting in an average OLR of 0.94 ± 0.23 g COD·L$^{-1}$·d$^{-1}$. A total of 61 batch cycles were run with the 100 L reactor. The 80 L reactor was run for 26 batches. During this time, a period of full-light spectrum illumination was studied in the 100 L reactor, by removing the UV-VIS absorbing foil from batches 13 to 37 (see Table 2 ). This allowed to study the influence of full-light illumination on the performance and stability of a PPB-enriched system. During this period, the 80 L reactor was kept as IR-illuminated control. We note, the reactor volume and the illuminated surface-to-volume ratio differed by 20%. The batch inoculation and reactor restart occurred as described in section 2.5.1. The produced biofilm was collected via vacuum from the walls (using a 1,250 W, 20 L wet vacuum with a stainless-steel collection drum Ozito). For each batch, samples of

the raw wastewater, initial suspended phase, final biofilm, and final suspended phase were collected for analysis. In addition, two detailed follow-up studies were carried out (in batches 33 and 53), taking samples every 3-4 h during the whole batch duration. This allowed to study the kinetics of the process and to assess the fluctuations during day-night cycles.

Table 2. Summary of the CWW test operating conditions for the 80 L and 100 L FPPBRs.

| | **Phase 1** | **Phase 2** | **Phase 3** |
|---|---|---|---|
| **Batch** | 1 - 12 | 13 - 37 | 38 - 61 |
| **Season** | Autumn | Winter | Spring |
| **Batch length (d)** | 3.6 (0.5) | 3.4 (0.7) | 3.4 (0.7) |
| **Light supply *** | IR | full spectrum | IR |
| **Acetic acid addition** | No | No | No |
| **VFA-COD at t=0 ($mg \cdot L^{-1}$)** | 65 (31) | 60 (46) | 227 (135) |
| **TCOD load per batch (g $TCOD \cdot L^{-1}$)**** | 3.2 (0.59) | 3.0 (0.47) | 3.4 (0.6) |
| **Irradiance ($MJ \cdot m^{-2}$)***** | 14.8 (3.2) | 13.5 (2.3) | 22 (4.7) |

* Only for the 100 L reactor, the 80 L reactor was illuminated with filtered sunlight and stopped after batch 26; ** the average TCOD:TN ratio was 19±0.7; *** calculated from daily global solar exposure data from the Bureau of Meteorology weather station 040082, around 2 km away from the PWW test location (http://www.bom.gov.au), measured from midnight to midnight.

### 2.6. Analytical methods

TCOD and SCOD, NH4+-N, NOx-N, NO2--N and PO43--P, VFAs, TKN, TP, TSS/VSS and TS/VS concentrations were determined as described elsewhere (Hülsen et al. 2018b). The ash content was calculated from the TS and VS measurements. The pH was measured by a HI 83141 portable pH meter (Hanna Instruments) (PWW tests) and a Mettler-Toledo M200 transmitter coupled to an Easysense pH 31 Mettler-Toledo

Limited, Port Melbourne, Australia (CWW tests). Temperature and DO concentrations were measured with an EasySense O2 21 DO sensor coupled to an M200 easy multiparameter transmitter (Mettler Toledo) and logged on a Site-Log LFC-1 4-20 mA Current Datalogger (Ocean Controls, MED – 005). Elemental analysis was performed by inductively coupled plasma optical emission spectrometry (ICP-OES) after 10% nitric acid digestion (Perkin Elmer with Optima 7300 DV,Waltham, MA, USA).The biomass crude protein (CP) content was calculated as particulate TKNx6.25, following Eding et al. (2006). Quantitative amino acid analysis and total carotenoid and total bacteriochlorophyll (BChl) contents of the harvested biomass were determined as described in Delamare-Debouteville et al. (2019) and Hülsen et al. (2020).

For the PWW tests, irradiance and temperature data were obtained from the Australian Bureau of Meteorology webpage for the University of Queensland Gatton station, ~2.3 km away from the reactor location. The Brisbane Port Control station, located 3.0 km away from the CWW industrial site, was used to get the irradiance values for these (experiments).

**2.7. Microbial analysis**

Microbial samples were harvested from the reactor liquid or directly from the biofilm. Biofilm samples were scraped off the reactor wall after draining. Raw samples were submitted to Australian Centre of Ecogenomics, The University of Queensland, for DNA extractions and paired-end 16S rRNA amplicon sequencing with primer sets 926F (50AAACTYAAAKGAATTGRCGG -30) and 1392wR (50-ACGGGCGGTGWGTRC-30) (Kunin et al. 2010). Miseq Sequencing System (Illumina, USA) targeting V6-8 was used. Data analysis was performed following (Hülsen et al. 2018b).

### 2.8. Data processing and statistical analysis

Statistical analyses were performed using the software R 3.5.0 (2019). When the distributions of the presented values were normal (verified with Shapiro-Wilk tests) and the homogeneities of variance were confirmed (using Bartlett's tests), ANOVA tests were used to assess significant differences, applying post-hoc Tukey HSD tests for comparisons. Otherwise, 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 same software was also used to determine correlations and linear regressions between variables. This analysis was performed using the results from the experiments with CWW, as the amount of data generated was sufficient. The package "corrplot" was used to create correlation matrices. The boxplots 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. Inputs are represented as averages and variability in inputs expressed as standard deviation in time-series measurements, represented as $\bar{X}(s_{X_i})$, where $\bar{X}$ is the average value for the data $X_i$, and $s_{X_i}$ is the standard deviation for the data. Outputs and calculated parameters (including slopes from linear models) are represented as average value, with uncertainty expressed as uncertainty in mean based on a two-tailed *t*-text (95% confidence, 5% significance threshold), represented as $\bar{X} \pm E_{\bar{X}}$, where $E_{\bar{X}}$ is the 95% confidence interval. Day and night removal rates for the cycles studies were calculated between each measuring point pair.

The TCOD removal and recovery were calculated as shown in equations (Eq.) 1-4. TN and TP removals and recoveries were also determined with these equations.

$$TCOD_{removed\ (\%)} = 100 - \left(\frac{TCOD_{final,out}\ (mg\ L^{-1})}{TCOD_{start,in}\ (mg\ L^{-1})}\ x\ 100\right) \text{(1)}$$

$$TCOD_{recovered\ (\%\ of\ TCOD_{start,in})} = \frac{TCOD_{harvested}(mg\ L^{-1})}{TCOD_{start,in}\ (mg\ L^{-1})}\ x\ 100 \text{ (2)}$$

$$TCOD_{harvested}\ (mg\ L^{-1}) = \frac{Areal\ productivity\ (gCOD\ m^{-2}d^{-1})\ x\ batch\ length\ (d) * illuminated\ surface\ (m^{-2})}{Reactor\ Volume\ (L)}\ x\ 1000 \text{ (3)}$$

$$TCOD_{recovered\ (\%\ of\ TCOD_{removed})} = \frac{TCOD_{harvested}(mg\ L^{-1})}{(TCOD_{start,in}\ (mg\ L^{-1}) - TCOD_{final,out}\ (mg\ L^{-1}))}\ x\ 100 \text{ (4)}$$

# 3. Results and discussion

## 3.1. Reactor start-up and PPB biofilm formation

Without inoculum and mixing, PPB growth had fully covered the walls after 25 d. This demonstrates selection and enrichment of native PPB from high N concentration PWW ($NH_4^+$-N > 1,000 mg·$L^{-1}$) as well as (slow) light-driven attached biofilm formation (thickness ~2-4 mm) (Figure 2A). With mixing, biofilm formation and enrichment of PPB from PWW was reduced to 6 days, indicating the relevance of substrate supply and the possibility of fast start-up, as previously reported for suspended PPB cultures (Hülsen et al. 2018a). The same was observed for CWW (Figure 2B), where start up with mixing occurred in 6 days, forming thick purple biofilm on the inner reactor walls (Figure 2C).


Figure 2: PPB grown as attached biofilm on (A) PWW and (B) CWW, and (C) a representative picture of the attached PPB biofilm on the inner reactor wall (CWW). In each case, sections where biofilm has been harvested can be seen.

### 3.2. Wastewater treatment performance

#### 3.2.1. Piggery wastewater - COD, N, & P removals and biomass productivities

The attached biofilm formation of PPB and the associated TCOD, TN and TP removals were impacted by COD bioavailability (particularly VFA) and light supply (shading), both limiting the maximum achievable removal rates. This was observed in the 80 L and 100 L PBRs, with different surface/volume ratios but similar organic and nutrient removal efficiencies ($p>0.24$ and 0.19) over the periods (see Figure 3). An overall increase in

performance (COD, N and P removals efficiencies) from phase 1 to 3, coincided with increased in VFA content (naturally and due to acetic acid addition) in the wastewater (Figure 3A-D). Both PBRs slightly increased the TCOD removals at increased VFA concentrations, while the SCOD removal doubled to around 40% during phase 2. This was also observed for the 60 L unit, which however removed less TCOD and SCOD, with substantial fluctuations during Phase 1 (24.5±12% and 10.1±10.8%) and Phase 2 (30±33% and 41±65%). Similarly, a shortening of the batch lengths (from 6.6 d to 4.5 d) in phase 3 did not impact the COD removal efficiencies, indicating that the VFA-COD availability rather than batch length determined the removal performances in the reactors. Naturally, improved SCOD assimilation resulted in increased N and P uptake. Due to the high solid contents in PWW (particulate TKN, TP, COD and TS concentrations of 260 (372), 90 (70), 2,540 (1,675), and 2,420 (1,340) $mg \cdot L^{-1}$, respectively) release of soluble species and their uptake occurred simultaneously. Particulate TKN and TP was mobilised as $NH_4^+$-N and $PO_4^{3-}$-P, while both species were also assimilated. Therefore, TN and TP removal efficiencies are provided rather than ammonia/phosphate removal efficiency. The pH in the 100 L reactor at $T_{final}$ (similar for the 80 L reactor) decreased from phase 1 to 3 (phase 1; 8.1 (0.23), phase 2: 7.7(0.28) and phase 3: 7.0(0.32) but remained above the critical lower pH of ~6.0 for PPB (Puyol et al. 2020).

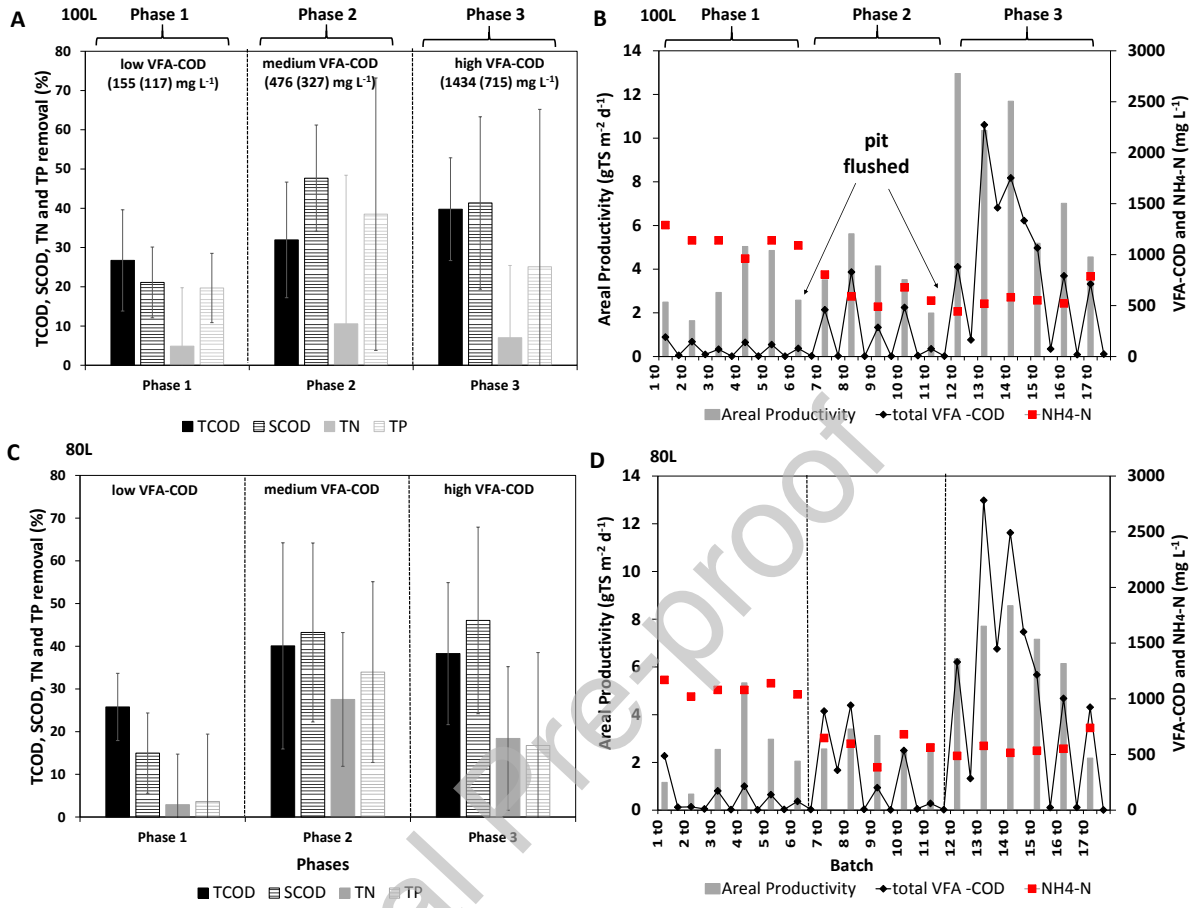


Figure 3: PWW test: TCOD (solid, black), SCOD (striped, black), TN (solid, grey) and TP (striped, grey) removal efficiencies (A) in the 100 L (C) and 80 L FPPBRs treating piggery processing wastewater (removal is based on $T_0$ and $T_{final}$ concentrations). PPB biomass areal productivities (grey bars), VFA at $t_0$ and $t_{final}$ (◆) and starting $NH_4^+$-N concentrations ( ) for (B) the 100L and (D) the 80 L FPPBR are also shown. Error bars represent confidence intervals. Phase 1 stands for the Australian spring, Phase 2 for summer, and Phase 3 for the autumn months.

With increased VFA-COD concentrations and shorter batch lengths during Phase 3, the attached PPB biofilm formation (as areal productivities) also increased. The $t_{final}$ VFA-COD excess during this phase further indicated oversupply (Figure 3B-D), as well as light limitations. Results from Phase 3, under non-limited VFA-COD conditions, give an indication of the capacity of the system, with COD removal rates up to 0.52 and 0.45 g $COD \cdot L^{-1} \cdot d^{-1}$ for the 100 L and the 80 L PBRs, respectively. Peak areal productivities reached 13 and 8.5 g $TS \cdot m^{-2} \cdot d^{-1}$ (or 9.5 and 6.2 g $VS \cdot m^{-2} \cdot d^{-1}$) in the 100 L and 80 L PBRs. These values are comparable to attached productivities achieved in continuous artificial illumination studies and on synthetic wastewater (Delamare-Debouteville et al. 2019, Hülsen et al. 2020). The enhanced performance of the 100 L over the 80 L reactor can be attributed to the higher reactor volume, which increased the COD availability per illuminated surface unit.

The results indicate that the performance was not impacted by high ammonium concentrations, peak global horizontal irradiance, batch length and reactor temperature. PPB were enriched at ammonium concentrations over 1.0 g $NH_4^+$-$N \cdot L^{-1}$ (~250 $mg \cdot L^{-1}$ of free ammonia at pH 8.2 and ~45 °C) and maintained the performance throughout Phase 1 without any effect on PPB growth and biofilm formation (noting that productivity was limited by VFA availability). This indicates an adaptation to high $NH_4$-N concentrations as opposed to a culture of non-adapted PPB, where a 5-fold decrease in specific activity was observed at 1,300 mg$NH_4$-N $L^{-1}$ relative to 100-500 mg $L^{-1}$ (Puyol et al. 2020). Regarding temperatures, the PPB culture routinely withstood liquid temperatures higher than 50 °C (up to 55 °C in phases 2 and 3, during summer and autumn). During the night, the temperatures decreased to ~20 °C, resulting in day/night temperature

fluctuations between 20-30 °C in the liquid (as shown in Figure S1A and Figure S2). The resistance of PPB to high temperatures (and wide daily variations) implies that cooling considerations and related equipment can very likely be omitted in PPB reactors (opposed to algal systems). The areal productivities in both FPPBRs increased despite a decrease in average peak global horizontal irradiances over the phases (Phase 1:, 23.8 $MJ \cdot m^{-2}$, Phase 2: 20.5 $MJ \cdot m^{-2}$ and Phase 3: 16.9 $MJ \cdot m^{-2}$, respectively). This confirmed that VFA-COD availability was the limiting factor, as previously reported (Hülsen et al. 2016b), until there is an excess of available COD, which makes light supply rate limiting.

In terms of reactor design, the results indicate that 100 mm wide flat plate reactors modules (100 L; 50 mm of average light path considering illumination from both sides) can be used effectively to produce PPB biomass (*e.g.*, highest peak productivity) and reduce the number of required reactor modules (due to an increased reactor volume) by 20% and 40% (comparing the 100 L with the 80 L and 60 L PBRs, with widths of 80 and 60 mm), which is desirable for wastewater treatment applications. This does not necessarily translate into capital savings, but implies that the mixing energy can be considerably reduced, as the same recycle flow rate was used for the 100 L, 80 L and 60 L reactors. In fact, at 60 mm reactor width (60 L PBR), the excessive shear due to mixing was counterproductive for biofilm formation, substantially lowering the attached areal productivities in the 60 L FPPBR (average of 2.0 ± 0.7 g $TS \cdot m^{-2} \cdot d^{-1}$) by 25% and 43%, compared to the 80 L and 100 L FPPBRs (see Figure S3), albeit an increased illuminated surface-to-volume ratio. We note that the mixing cost has to be reduced substantially to achieve feasible full-scale applications. This might also enable the use

of 60 mm wide FPPBRs, which however is less desirable for wastewater treatment applications (due to the volume).

### 3.2.2. Chicken processing wastewater - COD, N, & P removals and biomass productivities

The treatment performance of the 80 L and 100 L FPPBRs treating CWW wastewater improved moderately compared to the PWW tests. The TCOD, TN and TP removal efficiencies in the 80 L and 100 L FPPBRs treating CWW were on average 45 ± 2.5%, 26 ± 2.7% and 20 ± 2.9%, respectively (Figure 4A-C) (compared to TCOD, TN and TP removals of 18.5 ± 3.7%, 6.0 ± 1.9%, 23.5 ± 6.4% in the PWW tests). As for PWW, the removal performance in the 80 L and 100L FPPBR treating CWW were not significantly different (p-values > 0.57; the performances actually were slightly improved in the 100 L reactor, although not statistically significant due to high deviations). This confirms the benefit in increasing reactor width (to 100 mm) to increase the volume by 20%. This was confirmed during Phase 1, where both FPPBRs were illuminated with filtered sunlight (IR spectra) and achieved similar performances (batches 1-12; p-values > 0.23). The similarity persisted after removing the UV-VIS absorbing foil for 86 days (batches 13 – 37) from the 100 L reactor, using the 80 L as IR control (p-values > 0.23). This suggests that the removal performances can be maintained when full-spectrum sunlight is used as energy source (see section 3.2.2.1 for detailed cycle studies), albeit a 20% volume increase and a 20% illuminated surface-to-volume ratio decrease. However, green colour, indicating the presence of chlorophylls (from cyanobacteria and microalgae), developed over the course of Phase 2 in the 100 L FPPBR (starting after 2 weeks) (see Figure S4A-B). Despite the intense green coloration, PPB were still present

in considerable proportions in the reactors (see section 3.4). Microalgae might have acted as biological VIS-light filter, causing a layered biofilm structure (only visual evidence) and mutualistic/commensalistic relationships between PPB and algae (*e.g*. algae consuming $CO_2$ produced by PPB) (light measurements were not possible due to absence of submergible IR/VIS sensors; see Figure S4A-C for a scheme of the proposed layered biofilm).

As for the PWW wastewater tests, VFA-COD appeared to be limiting in the CWW tests. The $t_0$ VFA-COD concentrations were generally below 100 $mg \cdot L^{-1}$, with $t_{final}$ concentrations below 50 $mg \cdot L^{-1}$ (Figure 4B-D). The main difference in these experiments is that CWW was more biodegradable than PWW, and while containing similar amounts of solids (2.7(0.4) g $TS \cdot L^{-1}$ and 1.8(0.3) g $VS \cdot L^{-1}$), hydrolysable fats and proteins were mobilised in-situ (in the reactor) over the batch period (as opposed to PWW, anaerobically stored for weeks in the pit and still low on VFAs). As the CWW was directly fed in the FPPBRs, the released VFA-COD was not measured, but it likely provided a constant source of bioavailable COD, where hydrolysis, acidogenesis and acetogenesis was performed by an established anaerobic consortium, formed by PBB and anaerobic hydrolysers/fermenters (see Section 3.4 for a more detailed discussion of the microbial communities).

The higher COD availability with CWW is also suggested by the attached areal productivities, which were comparable (or higher) than those from the PWW tests with acetic acid addition in Phase 3 (Figure 4B-D), and higher than those achieved in VFA limited phases 1 and 2 of the PWW tests. Over the course of the experiment, the average attached (biofilm) and suspended biomass productivities differed significantly

between the 80 L and 100 L FPPBRs ($p < 0.0006$). The 80 L reactor (only Phase 1) generated on average 5.7 ± 0.9 g $VS \cdot m^{-2} \cdot d^{-1}$ and 11 ± 1.4 g $VS \cdot m^{-2} \cdot d^{-1}$ for attached and suspended biomass, while the 100 L reactor produced 8.7 ± 1.2 g $VS \cdot m^{-2} \cdot d^{-1}$ and 14 ± 1.7 g $VS \cdot m^{-2} \cdot d^{-1}$ (Phases 1-3). Interestingly, the productivities were similar during Phase 2 (batches 13-37), when the UV-VIS absorbing foil was removed from the 100 L reactor (p-values > 0.1). During this phase, the productivities decreased in both reactors, which was probably related to decreasing solar irradiances (winter started in cycle 16) (Figure 4B-D). This was further underlined by an increase of the attached productivities during Phase 3 in the 100 L, when the irradiance increased again due to seasonal changes (see Table 2, Figures S1 and S2).

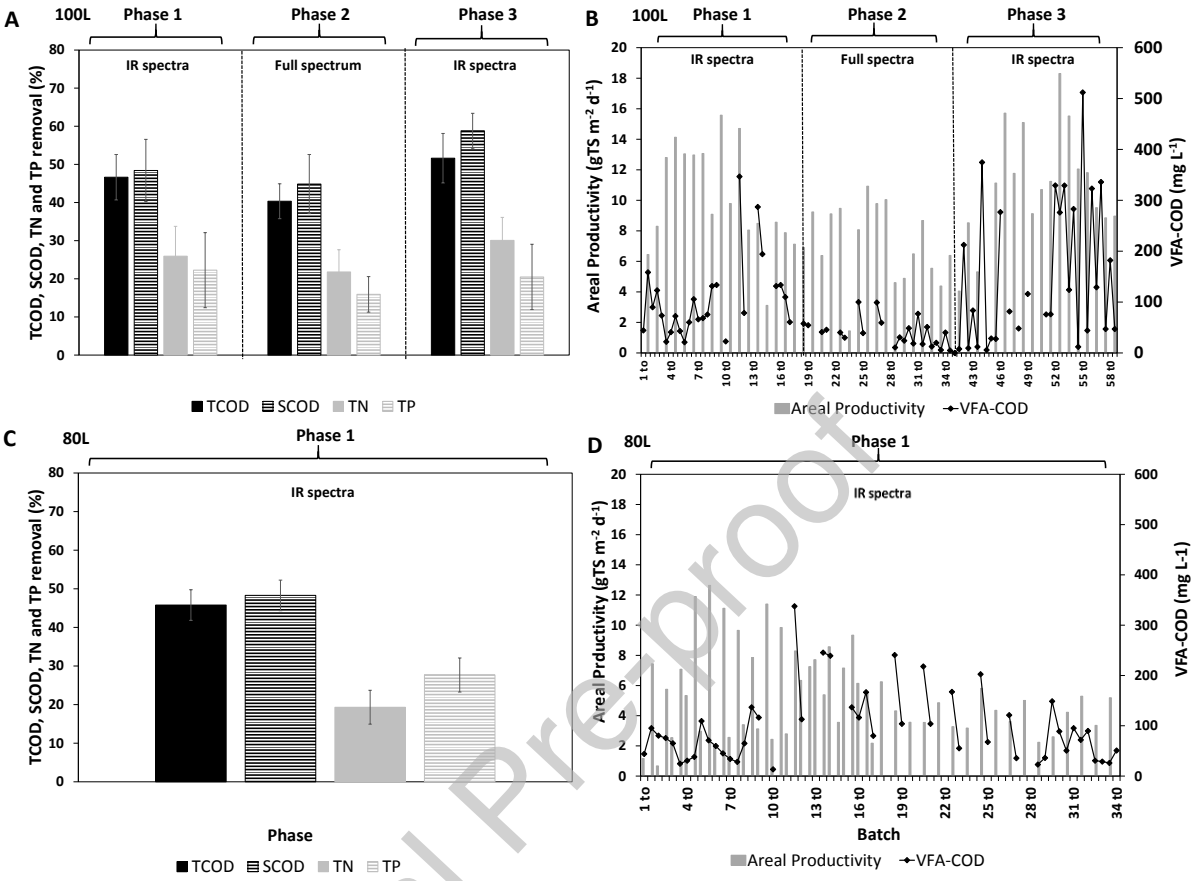


Figure 4: TCOD (solid, black), SCOD (striped, black), TN (solid, grey) and TP (striped, grey) removals of the 100L (A) and 80L (C) FPPBRs treating chicken processing wastewater and areal PPB biomass productivities (grey bars), VFA at $t_0$ and $t_{final}$ (◆) of the batches for the 100L (B) and the 80L FPPBR (D). Phase 1 stands for the Australian autumn, Phase 2 for winter, and Phase 3 for the spring months.

Correlation analysis of inputs and performance metrics could be done across the 80 cycles. For this number of observations, a Pearson correlation of >0.27 represents a weakly significant relationship ($p<0.05$), >0.34 a significant relationship ($p<0.01$), and

>0.4 a strong relationship ($p<0.001$). The corresponding correlation matrix is shown in Figure 5. For the cycle length (d), this variable was negatively correlated with the OLR (by definition considering constant influent composition) and with the productivities (suspended and total). Removal efficiencies were not correlated with the cycle length (or the OLR), which indicates that the batch duration could have been further reduced, allowing for a higher substrate load. In agreement with this, the biomass productivities (suspended, attached and total) were positively correlated with the OLR, indicating that higher values can be achieved by simply increasing the substrate load.

The irradiance and the temperature were also correlated, as both depend on the weather conditions and the incident sunlight. More importantly, the irradiance was positively correlated with the SCOD removal, the TCOD removal and the attached productivity, indicating that it had a significant effect on both PPB growth and biofilm attachment. This suggests that light limited biofilm formation, which would imply the occurrence of a light-driven biofilm, instead of the common shear-driven considerations. We reported similar observations in earlier works (Hülsen et al. 2020).

To identify if the correlation between temperature, irradiance and productivity was due to thermal effects or irradiance, a separate correlation analysis (due to lower number of data available, $n \approx 30$) using the reactor liquid temperature was performed. This identified that removal efficiencies and productivities were not correlated with liquid temperature ($\rho < 0.2$), and hence irradiance was the primary factor. Low temperatures have been previously identified as not substantially influencing the viability PPB-enriched cultures (Hülsen et al., 2016a). Generally, temperature fluctuations are expected to impose additional selection pressures across the microbial community in

outdoor environments, selecting for temperature resilient microbes. Considering the minimum and maximum liquid temperatures during the treatment period, ranging between 6.0 ºC (night during winter) and 47 ºC (daytime during summer) (see Figure S1B), the results confirm the resilience of PPB to temperature fluctuations, as described for the PWW tests.

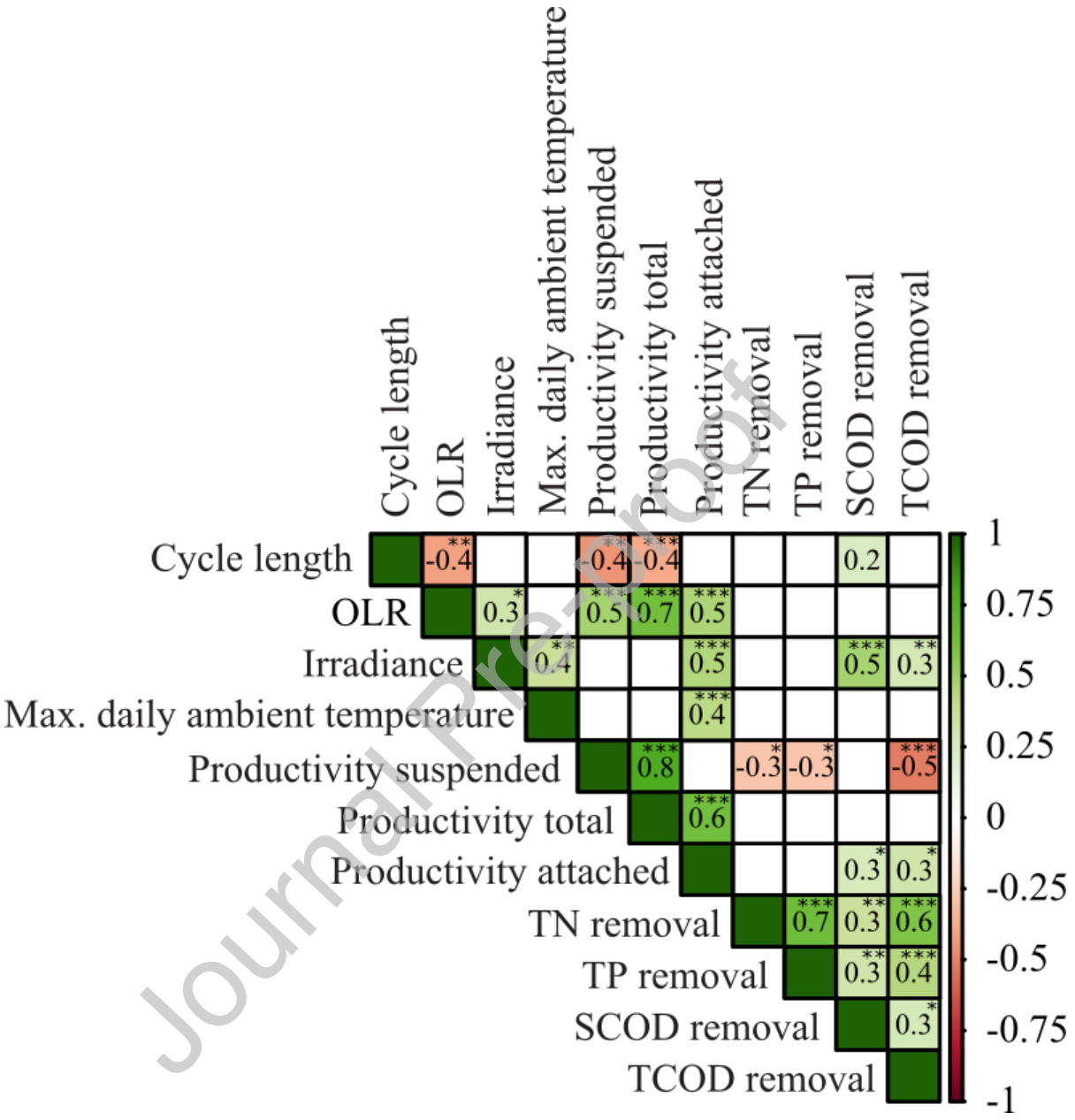


Figure 5: Correlation matrix using data from both reactors. Productivities are expressed in g $VS \cdot m^{-2} \cdot d^{-1}$, cycle length in days, OLR in g $COD \cdot L^{-1} \cdot d^{-1}$, removal efficiencies in percentage respect to the initial concentrations, temperatures in ºC, and irradiances in

$W \cdot m^{-2}$. The numbers correspond to Pearson correlation coefficients (ρ). * indicates p-values below 0.05, ** below 0.01, and *** below 0.001.

Other than being positively correlated to the OLR, both the attached and suspended productivities were correlated with the removal efficiencies. In the case of the attached productivity, a positive correlation was found with the TCOD and SCOD removal efficiencies, which is logical, as the removal in the system occurred due to biomass growth and attachment. The suspended productivities were negatively correlated to the removal efficiencies (TCOD, TN and TP). This might be partly due to biofilm formation and subsequent reduced light penetration, but mainly due to the dependence of total removal and biofilm attachment. This implies that, other than biomass growth, the removal efficiencies were limited by biomass attachment (*i.e.*, light attenuation due to the biofilm). In other words, more suspended biomass implied less attachment, and lower removal efficiencies (without biomass recovery). Further research should focus on maximizing biofilm formation, which appears to be limited by light.

#### 3.2.2.1. Day-night cycle studies

To study the kinetics of the process and to assess the fluctuations during day-night cycles, three sequential day-night periods were studied in detail in cycles 33 and 53. Both were done to evaluate and compare the kinetics under filtered and non-filtered illumination. Only the study starting in cycle 33 is shown here (cycle 53 is provided in Figure S5). The 80 L reactor served as UV-VIS-filtered control, while the 100 L reactor was illuminated with full-spectrum, unfiltered light. The results are presented in Figure 6. The behaviour of both reactors was similar ($p>0.05$). Both reactors were impacted by the day-night cycles. During the day, SCOD (mainly in the form of VFAs) was consumed

by PPB, leading to an increase in the pH and a concomitant TN and TP drop (assimilation of $NH_4^+$-N and $PO_4^{3-}$-P). The maximum SCOD removal rates were 810 and 880 mg $COD \cdot L^{-1} \cdot d^{-1}$ for the 80 L and 100 L FPPBRs. The SCOD:TN:TP ratios over the course of the batch were 100:5:0.6 and 100:7.5:1.1 for the 80 L and 100 L FPPBRs, respectively, which are well in line with those from the literature (Capson-Tojo et al. 2020). During the night, phototrophic activity ceased due to the lack of light, and VFAs were produced by fermentative bacteria (and to some extent also by PPB), leading to a pH drop and a decrease in the SCOD uptake rates or even mobilisation (Figure 6C-F). We note that continuous day and night COD mobilisation likely took place, resulting in continued VFA mobilisation and immediate uptake by PPB during the day, which also resulted in continuous SCOD removal over the batch length.

The TCOD removal rates, mostly due to biomass attachment and fermentative losses, varied substantially (Figure 6 C, F) over time. Despite the high variability, the SCOD and TCOD removal rates suggest that SCOD removal was mainly caused by assimilation and subsequent biomass attachment to the walls. The daytime TCOD removal rates serve as indicative rates of biomass attachment. A lower TCOD removal rate during the night, is indicative of the rates of fermentative COD removal and residual biomass attachment. Nevertheless, PPB consumed these compounds at a far higher rate during daytime. This mix of anaerobic fermenters and PPB as dominant clades is consistent with the identified microbial communities (see Section 3.4.

The illumination with full-spectrum light (and the presence of microalgae) did not significantly affect the removal kinetics nor the pH trend in the 100 L reactor when compared to the 80 L reactor (acting as control PBR illuminated with filtered light). In

addition, the DO concentrations were always below detection limits in both systems, indicating that the oxygen produced by microalgae was consumed immediately by facultative aerobes (or PPB). This also agrees with the microbial data, showing that aerobes appeared in small proportions in the 100 L reactor (e.g. *Pseudomonas sp*. on average <0.5% relative abundance) during this period (Section 3.4). This further excludes an effect of the spraying system on the presence of oxygen. The reason for the final TN and TP decreases in the 100 L (days 2.4-3.0) is unknown, but chemoautotrophic removal can be excluded, as $NO_x$-N was not found in the system.

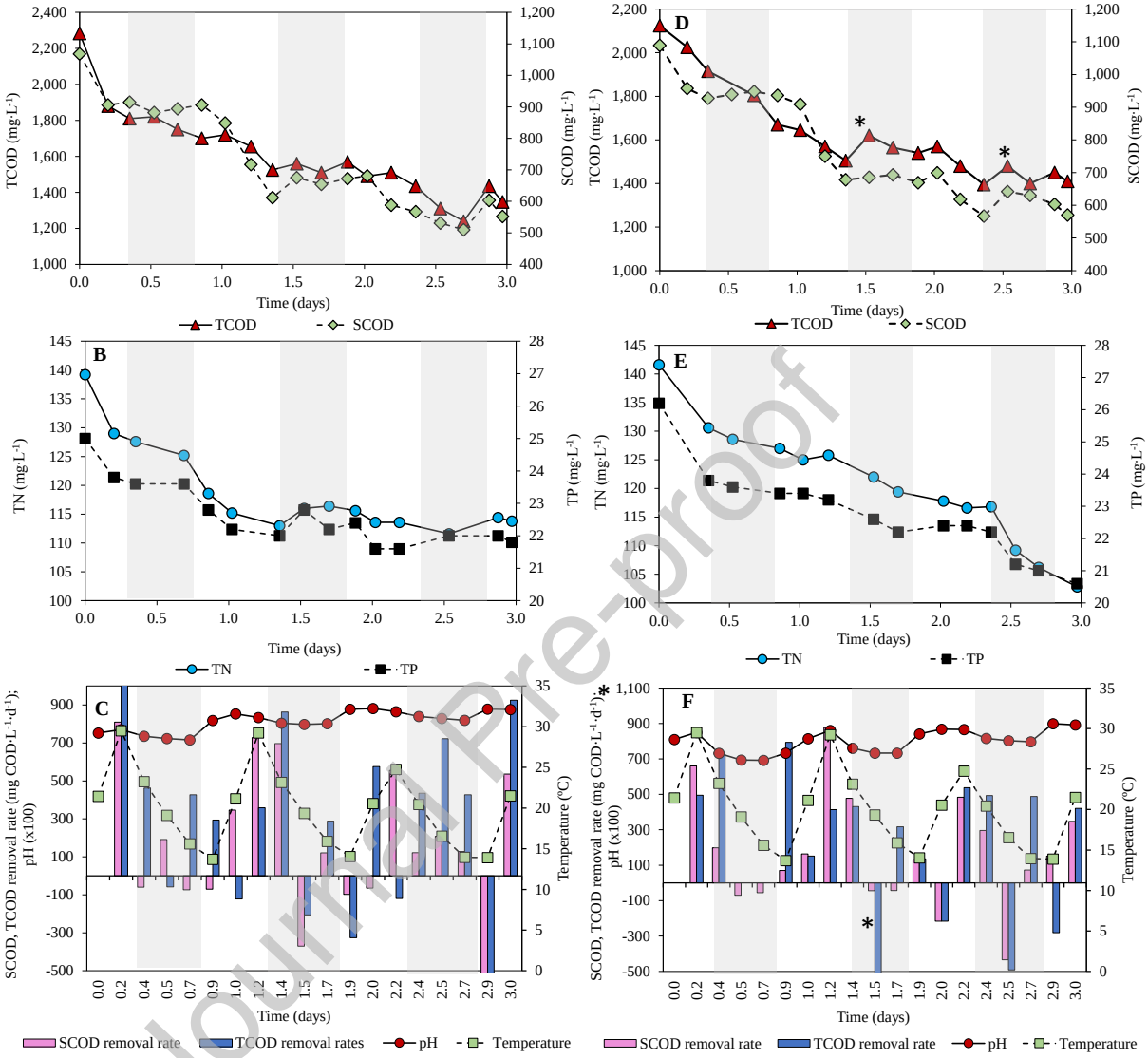


Figure 6: Evolution of the (A,D) TCOD and SCOD concentrations, (B,E) TN and TP concentrations and (C,F) SCOD and TCOD removal rates, pH and temperature during batch 33. The results of both the (A-C) 80 L reactor and the (E-F) 100 L reactor are shown. Night hours are marked as grey shaded areas. * indicates a sampling and/or measuring error (*e.g.*, due to sampling particulates with TCOD).

### 3.2.3. Resource recovery potential and biomass characteristics from PWW and CWW

The results indicate that, in both cases, resources could be effectively recovered, but was substrate (VFA) limited in the case of PWW, and illumination limited in the case of CWW. Recovery of COD, TN and TP in the PWW could be improved by acetate addition in phase 3. In this phase, the 100 L PBR recovered around 30% of the total COD, and 75% of removed COD as attached PPB biofilm (Figure 7A). This shows that bioavailable COD can be effectively partitioned from the bulk liquid into the biofilm. At the same time, the TN and TP recovery naturally increased with increasing COD recovery, but remained below 15% and 40% of the total TN and TP, respectively (Figure 7C) (TN is particularly low due to the high N influent concentration). This acts as a guide for the ideal substrate choice (optimal COD:N:P ratios) and identifies the need to preferment high nitrogen, particulate wastewaters. The COD, N and P recovery data for each batch in the 100 L and 80 L PBRs are shown in Figure S6 and S7.

For the CWW tests, the average total COD recovery was 30-40%, similar to Phase 3 from the PWW test (not VFA limited) (Figure 7B). The recovery efficiencies based on removed COD were also similar. The TN and TP recoveries were around 40% and 20%, consistent over the phases. The SCOD recovery based on harvested PPB biomass (*i.e.*, biofilm) was between 68-88%, and 132-180% based on removed COD, the latter confirming consistent SCOD release (from particulate organics) and non-measured uptake.

The attached recoveries account only for the harvested biofilm, excluding the separation/retention/harvesting of the suspended fraction. Harvesting the suspended

fraction would drastically improve the recovery efficiencies and would be suitable for wastewaters with low native solids concentrations. The increased recovery efficiencies when also harvesting the suspended fraction are obvious when looking at the attached and total biomass productivities in the reactors fed with CWW, increasing from ~10 g $TS \cdot m^{-2} \cdot d^{-1}$ (attached) to over 30 g $TS \cdot m^{-2} \cdot d^{-1}$ (total, attached and suspended) (Figure S8).

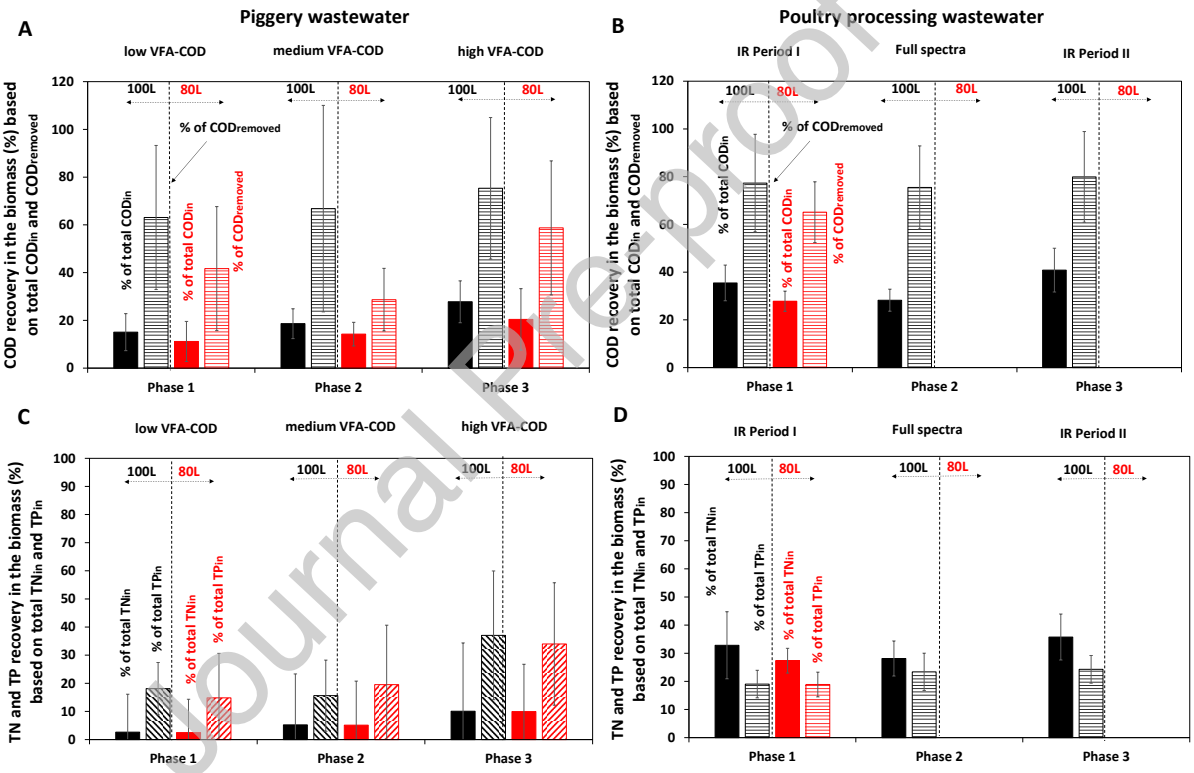


Figure 7: COD via harvested (attached) biomass from the 100L (black) and the 80L PBR (red) based on total $TCOD_{in}$ (at $t_0$) (solid) and TCOD removed (striped) treating (A) piggery (B) and chicken processing wastewater and TN (solid) and TP recoveries (striped) as biomass based on $TN_{in}$ and $TP_{in}$ (at $t_0$) (C and D) for both wastewaters. For PPW Phase 1 stands for the Australian spring, Phase 2 for summer, and Phase 3 for

the autumn months. For CWW Phase 1 stands for autumn, Phase 2 for winter, and Phase 3 for the spring months.

The biomass characteristics of the PPB biofilm harvested from the PWW and the CWW are shown in Figure 8. PPB biofilm from the PWW was harvested at average TS and VS contents of 99±5.1 g·L-1 and 71±4.0 g·L-1, resulting in an ash content of around 30% DM (dry matter). The TS, VS and ash contents of the harvested PPB biofilm varied significantly over time ($p<0.05$). The main reasons for that were the varying TS/VS ratios (0.77±0.45) in the wastewater and the harvesting procedure. After draining the reactor, particulates remained at the bottom. When harvesting the biofilm, this remaining layer had to be avoided manually, as it contained high amounts of ash, especially for PWW. It is possible that we occasionally harvested fractions of that layer, which might explain some of the variations. The variations in the TS and VS contents also contributed significantly varying CP, TN, TP, and heavy metal contents ($p<0.05$). The elemental composition of the dried and wet PPB biomass is presented in Table S2, which shows copper and zinc contents of 917±134 and 825±109 mg·kg TS-1 in the dried biomass. Overall, consistent biomass quality of the biofilm was not achieved over time and the Zn and Cu contents would only enable restricted agricultural use in Australia (Government 2018). Despite the high ash and heavy metal contents, the CP and amino acid contents, of 670±27 mg·g VS-1 and 497 g·kg VS-1, were high, and well in range with reported literature values (Hülsen et al. 2018a, Kobayashi et al. 1995, Ponsano et al. 2004, Shipman et al. 1975). See Table S3 for the amino acid profiles of the harvested biofilms. The harvested biomass further contained 8.7±2.7

mgcarotenoids·g VS-1 and 19.5±6.0 mgBChl·g VS-1, which was higher compared to the pigment contents in the suspended biomass (5.0±3.5 mgcarotenoids·g VS-1 and 10.7±4.6 mgBChl·g VS-1), confirming the PPB up-concentration in the biofilm. The relatively high concentrations of carotenoids and BChl in the harvested biofilm (of 618±192 and 1385±426 mg·L-1) show potential for pigment production (in a FPPBR in general). Overall, the pigment contents are well in line with those from mixed PPB culture biofilms in indoor, artificially illuminated, PBRs (Delamare-Debouteville et al. 2019, Hülsen et al. 2020), and on the higher end of reported suspended carotenoid and BChl ranges (Capson-Tojo et al. 2020).

For the CWW test, the harvested PPB biomass showed a much better and more consistent quality, mainly due to the lower mineral solid contents in the wastewater. The biofilm was harvested at average TS and VS contents of 88(3.6) g·L-1 and 81(3.5) g·L-1 respectively, consistent with the PWW biofilm. However, the ash contents were ~8.0% DM, substantially lower than in the PWW trials, and practically constant over the experiment. This also applies to the CP contents (>0.5 g·gdry-1 for 70% of the data).

As shown in Figure 8, a crucial advantage of the biofilm-based system is the high VS content in the biofilm when compared to the suspended biomass fraction (average values of 0.91-0.92 vs. 0.50-0.53 g·gdry-1). The high VS content in the biofilm might enable the application as high value-added product, particularly if already “clean” streams are used as influents (Hülsen et al., 2020). This is not possible with the recovered suspended biomass (i.e., via centrifugation), at least not for the here tested wastewaters (ash contents would be ~50%).

Overall, the attached biofilm productivities in the here described system are comparable to average yearly productivities in algal ponds and closed PBRs (6.8±3.0 and 9.3±2.0 g VS·m-2·d-1 (Richardson et al. 2014)). Volumetric productivities in our PPB system are higher compared to algal raceway ponds (0.035 g TS·L-1·d-1) but similar to algal flat plate and potentially tubular PBRs (0.27 and 0.56 g TS·L-1·d-1) (Jorquera et al. 2010), and also in line with other published works (Apel et al. 2017, Arbib et al. 2017, Eustance et al. 2016, Jorquera et al. 2010, Lee et al. 2014, Min et al. 2014, Slade and Bauen 2013, Tang and Hu 2016). We note that the values for annual averages have to be confirmed with long-term PPB system operations (no available data in literature yet). In comparison with attached productivities in algal biofilm systems (1.3–7.6 g TS·m-2·d-1) (Fica and Sims 2016, Gross et al. 2015, Johnson and Wen 2010), the areal productivities achieved with PPB are on the higher end of the reported range.

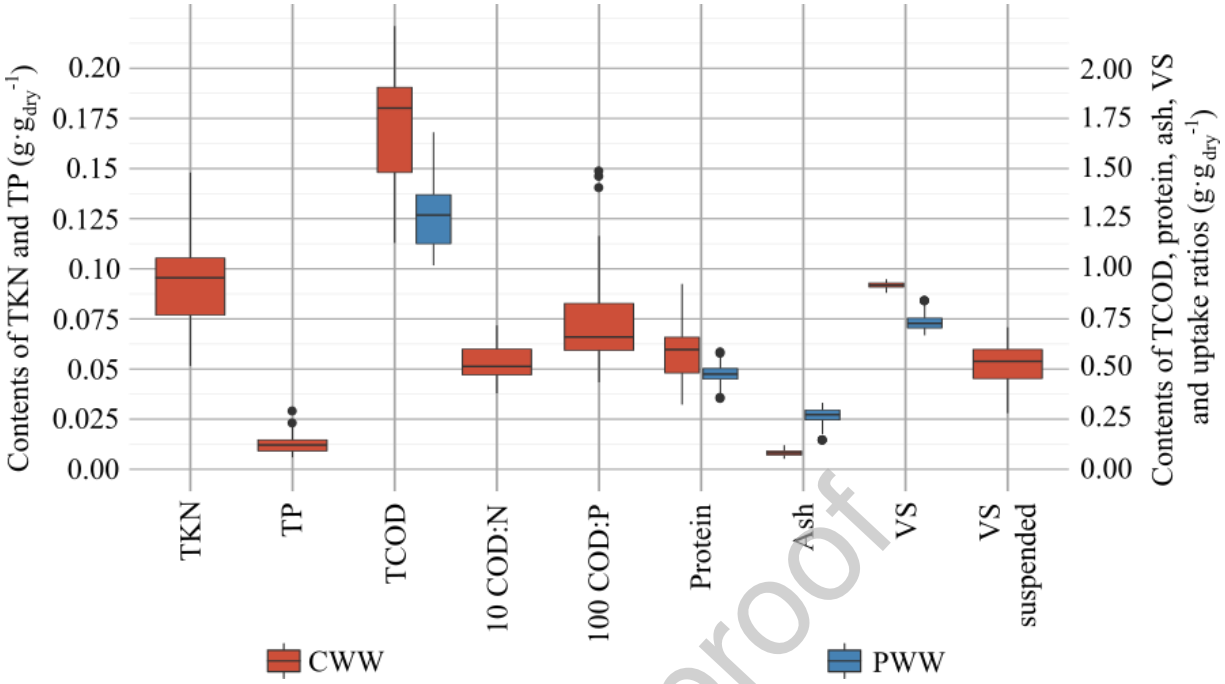


Figure 8: Characteristics of the dry collected biomass. All the values but the "VS suspended" (corresponding to the VS content of the suspended solids) refer to attached biofilm samples. The COD:N and COD:P uptake ratios correspond to the g of N per 10 g of COD and to the g of P per 100 g COD.

### 3.3. Microbial analysis of CWW and PWW tests

The microbial analyses confirmed the presence of a dominant PPB communities in the attached biofilm for PWW and CWW tests (Figure 9). The main PPB genus in the PWW tests were Rhodopseudomonas sp. (up to 57%) and Blastochloris sp. (up to 11%), while the CWW was dominated by Rhodobacter sp. (up to 27%) and Rhodopseudomonas sp. (up to 28%). A direct link to the wastewater cannot be made, and it seems more likely that the extended batch length in the PWW trials lead to the dominance of Rhodopseudomonas sp. This species has been found to dominate at long solid retention times (SRTs) in continuous reactors (Alloul et al. 2019, Hülsen et al. 2020).

For the PWW tests, the consistent presence of Rhodopseudomonas sp. in the attached biofilm and the suspension indicates its ability to adjust to prolonged exposure to thermophilic conditions, while comfortably switching to mesophilic conditions. While García et al. (2019) observed a steady decline of this genus when treating PWW in an open PBR (lab-scale, indoors), our results indicate that Rhodopseudomonas sp. seems well suited for outdoor applications. The presence of PPB in the suspended phase clearly shows the potential to improve the productivities when also harvesting this fraction (Figure 9B). This agrees with the pigment contents in the attached and suspended biomass. Vector plots and principal component analyses did not show sample clustering or significant microbial shifts over time (data not shown).

The flanking communities involved a mixture of common phyla in anaerobic digesters, as expected for an anaerobically digested influent: the VFA-degrading Firmicutes (e.g., Clostridia, Ruminococcaceae), Bacteroidetes (e.g. anaerobic fermenter Porphyromonadaceae), Euryarchaeota, Synergistetes, etc. These fermenters likely acted in a synergetic relationship with PPB, generating the VFAs to be consumed by the latter. On their side, PPB avoided pH drops and product-induced thermodynamic inhibitions.

For the CWW tests, the microbial analysis confirmed a dominant PPB culture in the harvested biofilm (suspension also had purple colour but was not analysed). The relative abundance of PPB in the 80 L and 100 L reactors ranged between 15-46% and 27-63%, with Rhodobacter sp. and Rhodopseudomonas sp. as predominant genera. In agreement with the decrease in biomass productivities, the abundance of PPB decreased during the periods of lower irradiance (samples from batches 17-33). Interestingly, while at the end of this period the PPB abundance in the 80 L reactor increased to 44% (cycle 33), this was not observed in the 100 L reactor. This is a consequence of the continuous decrease in PPB abundance that could be observed during the period illuminated with full-spectrum sunlight. As shown in Figure 9A, the PPB abundance decreased from 63% to 26%. While this decrease can be partially related to the lower

irradiances, lower light availability cannot explain by itself why the proportion did not increase at the end of the winter period (as in cycle 33 in the 80 L reactor) and, more importantly, it cannot explain why the PPB abundance increased to 48% after only 2 batches when the UV-VIS filter was placed again on the reactor (see Figure 9A). It is also striking that the dominant PPB genera changed from batch 33 to 35, from a mix of Rhodobacter sp. and Rhodopseudomonas sp. to Allochromatium sp. In batch 50, this dominance changed back to Rhodopseudomonas sp., and it can be expected that the previous distribution would establish itself is the coming batches. It can thus be concluded that the change from filtered to full-spectrum light resulted in lower PPB abundances. This was likely caused by a progressive out-competition of PPB by microalgae, as UV-VIS light was made available, e.g. due to small amounts of oxygen (which might lead to PPB outcompetition by aerobes (Capson-Tojo et al. 2021)). We exclude an effect of the surface spraying as this was done for both reactors over the course of the CWW tests. Surprisingly for the authors, the results of the microbial analysis did not show the increase in microalgae abundance. The primer sets used for amplification cover over 91% of the members in the phylum Chlorophyta (most common microalgae species), and have been previously used in similar studies (Hülsen et al., 2018b), so this was totally unexpected. Nevertheless, the intense green colour in the 100 L reactor at the end of the period illuminated with full sunlight clearly showed that microalgae were present in the PBR in considerable proportions (Figure S4).

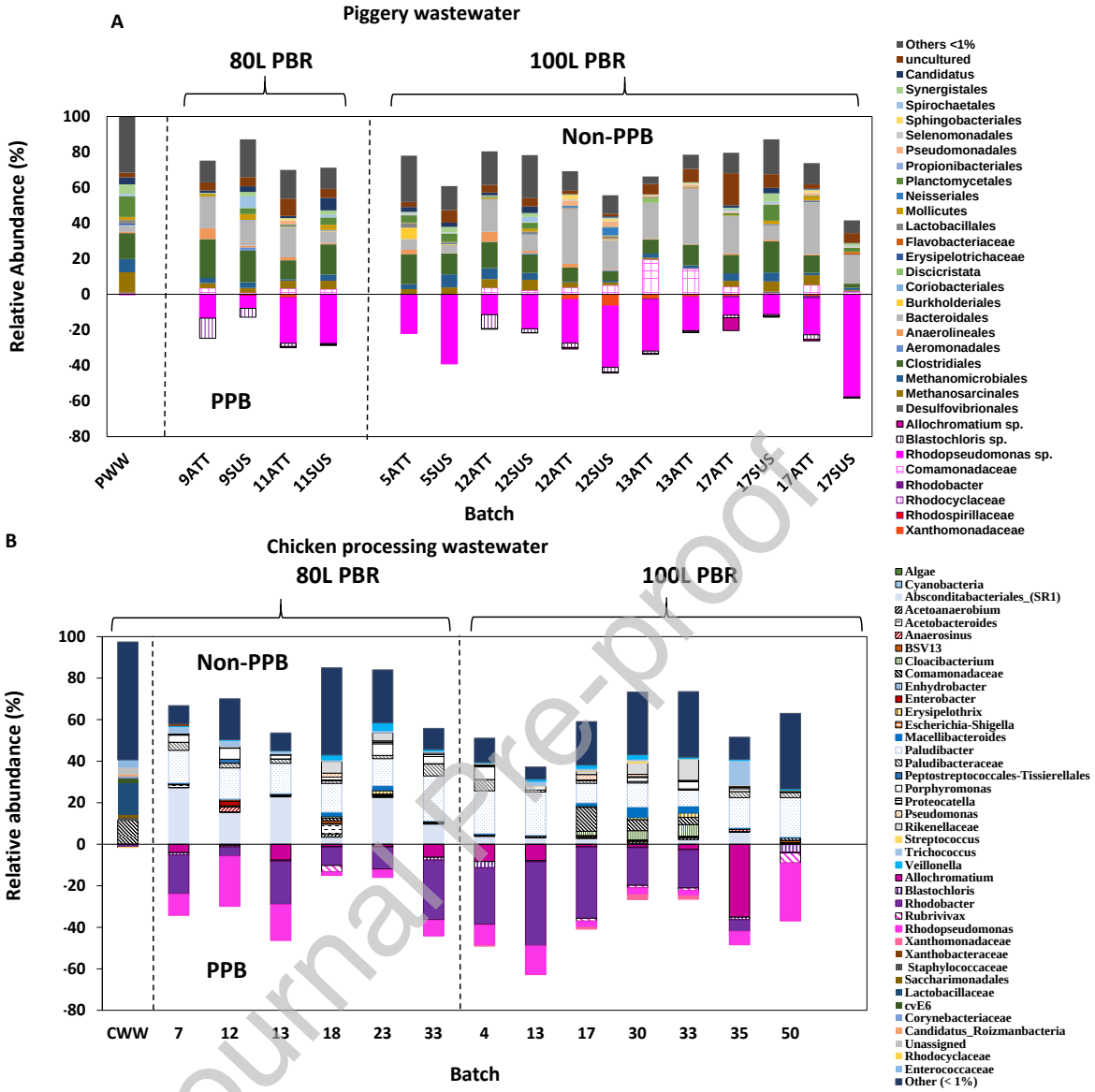


Figure 9: Relative abundances based on 16S analysis for the 100 L FPPBR (A) for the CWW test and (B) the PWW test. PWW and CWW represents the raw wastewater ATT and SUS stand for attached biofilm and suspended biomass samples.

### 3.4. Significance for industrial application

This study shows the technical feasibility of mixed PPB cultures in outdoor FPPBRs to treat two completely different, complex wastewaters under real conditions. COD, TN

and TP were removed via biofilm attachment, achieving removal efficiencies of up to 50%, 30% and 30%, respectively. These removals could be realised in a flow-through cell (e.g., plug flow), without additional biomass retention. This might enable shorter HRTs, and result in biomass attachment, while suspended biomass is washed out. A fraction of the resources could be recovered as PPB biofilm, while reducing the discharge costs, e.g., when discharging to the sewer. This would save both capital and operational costs (e.g., no membranes needed) but would minimise the overall removal and recovery. Assuming a retention step allowing the harvesting of suspended biomass, such as membrane filtration (e.g. 0.45µm (Hülsen et al. 2016b)) or centrifugation, the removal efficiencies of TCOD, TN and TP could be increased to 71-78%, 22-67%, and 37-65%. It is important to consider that PPB do not remove total COD, TN and TP, but merely assimilate soluble components into biomass. A harvesting step would consequently increase the areal productivities, from ~10 (attached) to over 30 g TS·m-2·d-1 (total) (or ~0.6 g TS·m-3·d-1). However, a large fraction would not necessarily be PPB biomass, especially for wastewaters such as the ones tested here, with high solid contents. The lower quality biomass from the suspended fraction could be used as fertiliser, but not for SCP applications, as the ash content would be around 50%, and the heavy metals contents would limit its application (especially for PWW). While the quality of the attached biomass (i.e., biofilm) can be consistent over time, with high CP contents (over 50%), its harvesting would require an automated system, which would add complexity and costs. However, this might be justified to enable consistent product quality in high value-added applications, such as SCP for fishmeal substitution in fish or

prawn feed (Alloul et al. 2021b, Delamare-Deboutteville et al. 2019), which currently sells at 1,400 USD·tonne-1.

Alternatively, improved product consistency and protein contents in a suspended or hybrid system could be achieved via a combination of wastewater pre-fermentation, and solids separation using off-the-shelf technologies (e.g., via dissolved air flotation). It is clear that an optimised PPB process requires VFAs, and constant VFA availability will also increase the PPB abundance, relative to in-situ fermentation. Comparison of the PWW vs CWW operation emphasises that this technology best fits readily degradable or fermented wastewaters. Food processing (including meat processing) is a ready target, particularly in combination of pre-fermentation. Production wastewater (with a significant manure component) is less optimal, but PPB could have a role in a combined system, where organic acids are produced in a leach bed, and PPB produced in a liquid side stream (Bayrakdar et al. 2018). Minimising solids in the feed would also enhance the quality of the suspended fraction. Depending on the local legislation, food grade substrates might be required to consider any application as SCP in feed which, e.g., in European Union, depends on the category of the feed material (EU 2002) (also see regulation (EC) No 429/2008). Application of wastewater-derived biological material, especially for feed, is not a straightforward process and requires a carefully considered legislative framework (Smedley 2013).

Dedicated wastewater pretreatment might enable continuous operation during the day (rather than batch operation), likely with shorter HRTs (e.g. ~1 d during 12 h), as previously reported in lab reactors (Hülsen et al. 2016a, Hülsen et al. 2016b, Hülsen et al. 2018b). In combination with biomass retention and continuous harvesting, this would

enable SRT control and potentially improve volumetric removals. The goal for future research should be continuous operation. However, the here reported COD removal rates of around 0.8 – 0.9 g COD·L-1·d-1 are likely to be close to the theoretical possible rates, assuming day/night cycles and outdoor conditions (in sunny Brisbane, Australia) at given design parameters (biofilm, 5 cm light path (10 cm width), 20 illuminated m2·m-3). We expect a suspended system to realise higher rates, due to reduced shading on the walls over time. The inherently higher recovery efficiencies and biomass productivities are another major advantage of suspended processes.

Another major conclusion from this study is the general robustness of the PPB PBRs. PPB withstood temperature and light variations seemingly unaffected. While light in the subtropical Brisbane, Australia is favourable, this also leads to system overheating during hot weather, e.g. in summer. With regular temperature peaks over 50 °C, we can conclude that cooling would not be required to maintain a functioning PPB culture. This is a major advantage compared to microalgae systems that have been reported to collapse around these temperatures (Mata et al. 2010, Tredici and Materassi 1992). This removes a major barrier to adoption by eliminating significant capital and operational costs, and water consumption previously associated with algae PBR systems (Béchet et al. 2010, Schenk et al. 2008). Temperatures ranging between 6.0 °C and 55 °C throughout the year did not have an observable effect on the performance. This robustness also to low temperatures was previously shown in lab-scale studies (Hülsen et al. 2016a), and are now confirmed in outdoor systems. The process robustness is further underlined by the fact that we did not rely on process controls.

The reactors were deliberately left open to atmosphere, which saves costs in a potential full-scale plant. As opposed to ponds, the surface for air diffusion is very small in FPPBRs, which reduces the oxygen transfer (also considering high COD concentrations). This is highly relevant for a stable, functioning PPB community (Capson-Tojo et al. 2021). In this context, the attenuation of IR light as opposed to VIS light is also different due to the increased absorption of IR wavelengths by water. Light attenuation in PPB cultures requires further research, to determine the optimal light path at given biomass concentrations.

Besides the batch operation at relatively long HRTs, the main operating costs of this system are the mixing energy, the biomass harvesting and dewatering, and the lack of light during night-time, complicating continuous operation. Low-cost mixing has to be realised, focussing either on the reduction of the liquid recycle ratio (*e.g.*, via nozzles) or on the implementation of static impellers (or similar). With recirculating liquid mixing, PPB production is prohibitively expensive. We note that low energy mechanical mixing solutions exist, but need to be optimised for narrow, long, and tall reactor systems. An automated harvesting system is also required, and manual harvesting is excessively labour intensive, even in countries where costs are low. The dewatering of the harvested biofilm is a lesser issue, but any harvesting of the suspended biomass will encounter problems similar to microalgae systems. Research using outdoors reactors has to focus on these aspects, applying reasonable sized PBRs.

## 4. Conclusions

Three reactor sizes (60, 80, and 100L) were operated in aggregate over 1.5 years on piggery production (PWW) and chicken wastewater (CWW) with a goal of resource recovery as biofilm and demonstration using natural light outdoors, and at relevant scale. The system could be operated stably on both wastewaters, in temperature ranges from 6°C-50°C, and with daily fluctuations of 20°C-30°C.

Performance was limited generally by availability of VFAs, with light being less of a limitation. Therefore, performance was generally better on the CWW feed, which had a higher degradability. The larger (100L) reactor generally performed best, with the 60L reactor performing poorly due to a lower volumetric capacity and to intense mixing.

Biofilm could be recovered at a high solids concentration (~90 g TS·kg$^{-1}$), and had a much lower ash and protein content (>50%), but the majority of biomass remained in suspension and had poorer properties, particularly for PWW. This means that a PPB system is better suited to highly degradable feeds and/or combination with pretreatment, particularly prefermentation.



## Declaration of Interest Statement

none

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