A systematic optimization of piggery wastewater treatment with purple phototrophic bacteria.

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Abstract

The increase in natural water bodies pollution caused by intensive animal farming requires the development of innovative sustainable treatment processes. This study assessed the influence of piggery wastewater (PWW) load, air dosing, CO_2/$NaHCO_3^-$ supplementation and pH control on PWW treatment by mixed cultures of purple phototrophic bacteria (PPB) under infrared radiation in batch photobioreactors. PPB was not able to grow in raw PWW but PWW dilution prevented inhibition and supported an effective light penetration. Despite the fact that PPB were tolerant to O_2, carbon recovery decreased in the presence of air (induced by stripping). CO_2 supplementation was identified as an effective strategy to maximize the removal of carbon during PPB-based PWW treatment with removal efficiencies of 72% and 74% for TOC and VFAs. However, the benefits derived from CO_2 addition were induced by the indirect pH control exerted in the cultivation medium. Thus, PPB supported an optimal pollutant removal performance at pH 7, with removal efficiencies of 75%, 39% and 98% for TOC, TN and VFAs.

Keywords: Nutrient recovery; PPB; Purple non-sulphur bacteria; Photosynthetic bacteria; Swine manure.

1. Introduction

The uncontrolled discharge of wastewaters is a severe environmental problem worldwide. The contamination of natural water bodies with organic matter, nutrients, pathogens and toxic pollutants causes eutrophication of surface waters and limits the potential uses of water (García et al., 2019; Godos et al., 2010). Wastewaters are typically classified according to their origin into domestic, agricultural, industrial and agro-industrial wastewaters. Piggery wastewater (PWW) is an agro-industrial wastewater characterized by its high content of organic matter and nutrients (Chen et al., 2018) as a result of the limited use of process water during intensive pig farming. The need for a decentralized and cost-effective treatment of these high strength effluents is fostering research on the development of innovative and sustainable solutions to cope with this increasing environmental problem in rural areas (Godos et al., 2009).

In the past years, biotechnologies based on bacteria or microalgae growth have been engineered to treat wastewater since they entail a lower energy consumption and higher potential to recover nutrients compared to physical/chemical wastewater treatment technologies (García et al., 2019; Godos et al., 2009; Hülsen et al., 2018b, 2014). However, while anaerobic digestion is only capable of recovering the carbon and energy in the form of biogas (i.e. CH_4 and CO_2), the high concentrations of NH_4^+ typically encountered in PWW severely inhibit the growth of both microalgae and anaerobic microbial communities (Yenigün and Demirel, 2013). Therefore, there is a need to develop new biotechnological platforms capable of maximizing carbon and nutrient recovery from PWW at reduced operating costs.

In this context, purple phototrophic bacteria (PPB) constitute an emerging biological platform for the treatment of high strength wastewaters (Puyol et al., 2017). PPB exhibit high growth rates, can grow at high salinity (Hülsen et al., 2019), exhibit a versatile metabolism and are tolerant to low temperatures. Indeed, PPB are capable of carrying out an efficient domestic wastewater treatment process at temperatures of 10 and 11 °C (Dalaei et al., 2019; Hülsen et al., 2016a). In this context, psychrophilic PPBs capable of growing at temperatures ranging from 0 to 25 °C have been isolated in the Antarctic (Madigan et al., 2000). Indeed, PPB can grow photoautotrophically and photoheterotrophically using light as energy source, and many forms of organic and inorganic compounds as electron donors. Likewise, PPB can grow chemoheterotrophically and chemoautotrophically using the energy from organic or inorganic compounds, respectively, and oxygen, nitrate/nitrite, or sugars as electron acceptors. PPB are capable of fixing carbon dioxide using phototrophic or chemoautotrophic metabolism, or using organic compounds as a carbon source under photoheterotrophic or chemoheterotrophic mode (Larimer et al., 2004). The phototrophic mechanism is remarkable as PPB use the near infrared range to power bacterial growth, which favors the selective cultivation of PPB (Hülsen et al., 2014). PPBs exhibit advantages compared to other photosynthetic microorganisms in terms of light utilization efficiency and tolerance to organic and nitrogen pollution. Thus, PPB have a higher conversion efficiency of photons (6-8%) than microalgae (< 5%) (Posten and Schaub, 2009). A lower radiation intensity is required for effective PPB cultivation (< 50 W m^{-2}) compared to microalgae (> 200 W m^{-2}) (Gordon and Polle, 2007; Suwan et al., 2014). Infrared radiation is attenuated to a lesser extent than visible light in the culture broth, which entails a greater penetration of IR in high strength wastewaters (Hülsen et al., 2018b). Finally, PPB exhibit a high tolerance to organic and nitrogen

pollution as a result of their versatile heterotrophic and mixotrophic metabolism (Lu et al., 2019b). Overall, PPB have been the dominant photosynthetic organisms in the mixed liquor of batch (Hülsen et al., 2018a) and continuous photobioreactors treating wastewaters under anaerobic conditions (Hülsen et al., 2018b). Most studies in literature have focused on the evaluation of the potential of PPB for carbon and nutrient removal in domestic wastewaters (Hülsen et al., 2016b, 2014), while the number of investigations assessing the optimization of the potential of PPB for the bioremediation of high-strength wastewaters such as PWW is very limited.

In this work, the potential of mixed cultures of PPB for the bioremediation of PWW was investigated in batch photobioreactors under infrared radiation. More specifically, the influence of the PWW load, air dosing, CO_2/$NaHCO_3^-$ supplementation and pH control on PPB growth and on carbon and nutrient removal from PWW was investigated.

2. Materials and Methods

2.1. Piggery wastewater and inoculum

The PWW, previously centrifuged in an industrial decanter, was collected from a swine farm at Narros de Cuéllar (Spain) and stored at 4 °C. The PWW was further centrifuged for 10 min at 10000 rpm. The composition of the resulting PWW was as follows: total organic carbon (TOC) concentration of 15775 ± 487 mg L^{-1}, total carbon (TC) concentration of 16922 ± 549 mg L^{-1}, inorganic carbon (IC) concentration of 1149 ± 223 mg L^{-1}, total nitrogen (TN) concentration of 5028 ± 339 mg L^{-1}, total suspended solids (TSS) concentration of 4.3 ± 0.3 g L^{-1} and pH 7.95 ± 0.1.

The mixed PPB community inoculum used was obtained from a batch enrichment in 10 fold diluted PWW with *Rhodopseudomonas* as the dominant genus with a 82% relative abundance (García et al., 2019). Fresh inoculum was prepared in 1.2 L gas-tight bottles containing 400 mL of 10 fold diluted PWW under a He atmosphere. The inoculum was incubated under magnetic agitation at 300 rpm and infrared radiation at 50 W m^{-2}.

2.2 Chemical and reagents

CO_2 (≥ 99.9%) and Helium (≥ 99.5%) were purchased from Abello Linde (Barcelona, Spain). HCl (~ 37%) and $NaHCO_3$ were obtained from Fisher Scientific (UK) and Cofarcas (Spain), respectively.

2.3. Batch PWW biodegradation tests

PWW biodegradation tests were performed batchwise in 1.2 L gas-tight glass bottles (Afora, Spain) in duplicate. The bottles were initially filled with 450 mL of the corresponding PWW and inoculated with 50 mL of fresh PPB inoculum. Unless otherwise specified, the bottles were then closed with butyl septa and plastic caps, and flushed with He for three minutes at a high flow rate in order to replace the air headspace with an inert atmosphere. An inert gas such as He without biological function was used to avoid any potential interference, since N_2 can be fixed by PPB under specific cultivation conditions. The batch tests were incubated under magnetic agitation at 300 rpm, 30 ± 2 °C and infrared radiation of 50 W m^{-2} with light-emitting diodes OSLUX® SFH 4780S and SFH 4715AS, centroid emitting at a wavelength 810 and 850 nm, respectively (OSRAM, Germany). A non-inoculated control test using 10 fold diluted PWW and prepared as above described was also always conducted in each test series. A sample of 5 mL of liquid culture was taken every two days to analyze culture

absorbance (samples were diluted with water in order to adjust the readings between 0.2 and 1.0), pH and the concentration of TOC, IC, TN and volatile fatty acids (VFA), while 100 µL of the bottle headspace was drawn with gastight syringes (Hamilton, USA) to quantify the gas concentration of CO_2, H_2S, CH_4. PPB growth was monitored using culture absorbance at 808 nm (OD_{808}), which represents a specific spectral niche for these phototrophic microorganisms compared to other phototrophic species (Stomp et al., 2007). Thus, although the organic matter present in PWW preferentially absorbs at wavelengths under 700 nm (Fig. S1), PPB mainly absorb with characteristic peaks above 800 nm (Hülsen et al., 2019), corresponding to bacteriochlorophyll *a* (Hunter et al., 2009).

2.3.1. Test series 1

The influence of PWW load on PPB growth and carbon and nitrogen removal from PWW was assessed in Test series 1 (Fig. S2) in order to elucidate any potential inhibition of PPB by NH_4^+ or organic pollutants present in PWW. For this purpose, undiluted PWW and 5, 10 and 15 fold diluted (in tap water) piggery wastewaters were incubated with PPB for 20 days (final stationary phase of growth) as above described.

2.3.2. Test series 2

The influence of air dosing on PPB growth and carbon and nitrogen removal from PWW was evaluated in Test series 2 using 10 fold diluted PWW in order to assess the bioremediation potential of PPB under aerobic, microaerobic and anaerobic conditions. Two tests were performed under an open air atmosphere to maintain aerobic conditions with and without PPB inoculum. PWW biodegradation tests inoculated with PPB under

168 a He atmosphere were also carried out with and without a periodic injection of 20 mL of
169 air every two days. The tests were incubated for 20 days.
170
171 *2.3.3. Test series 3*
172 The influence of the addition of CO_2 and $NaHCO_3$ on PPB growth and carbon and
173 nitrogen removal from PWW was evaluated in Test series 3 using 10 fold diluted PWW
174 in order to elucidate the enhancement in the bioremediation potential of PPB mediated
175 by an external CO_2 addition. PWW treatment by PPB was evaluated under a He
176 atmosphere in tests supplied every two days with 25 mL of pure CO_2 (≥ 99.9%) or with
177 1 mL of $NaHCO_3$ (8 g L^{-1}). A control test inoculated with PPB and incubated without
178 CO_2 or $NaHCO_3$ addition was also carried out. By day 22, 1 mL of trace elements
179 solution (López et al., 2018) was supplied to the control PPB test in order to assess if
180 PWW treatment was limited by trace metal availability.
181
182 *2.3.4. Test series 4*
183 The influence of pH and pH control strategy on PPB growth and carbon and nitrogen
184 removal from PWW was evaluated in Test series 4 using 10 fold diluted PWW in order
185 to elucidate whether the beneficial effect of CO_2 was due to its role as electron donor or
186 to its contribution to maintain the pH low. PWW treatment by PPB was evaluated under
187 a He atmosphere in tests supplied every two days with 25 mL of pure CO_2 (≥ 99.9%),
188 with HCl in order to match the pH of the CO_2 supplemented tests and with HCl in order
189 to maintain the pH at 7. A control test inoculated with PPB and incubated without CO_2
190 or HCl addition was also carried out.
191
192 *2.4. Analytical methods*

193 Dissolved TOC, TC, IC and TN concentrations were analyzed using a TOC-VCSH
194 TOC analyzer (Shimadzu, Japan) equipped with a TNM-1 unit. VFAs concentrations
195 were determined in a 7820A gas chromatograph (GC) equipped with a FID detector
196 (Agilent, USA) as described elsewhere (López et al., 2018). Samples for TOC/TN and
197 VFAs analyses were centrifuged at 10000 rpm for 10 min. The spectrum of absorbance
198 of PPB culture broth samples was analysed in a UV-2550 spectrophotometer
199 (Shimadzu, Japan) in the range at 350-850 nm. Gas concentration of CO_2, H_2S and CH_4
200 in the headspace of the bottles was determined using a CP-3800 GC equipped with a
201 TCD detector (Varian, USA) according to García et al. (2019). A pH 510 pHmeter
202 (Cyberscan, Netherlands) was used for pH determination. Finally, the quantification of
203 TSS concentration was performed at the beginning and end of each test series according
204 to Standard Methods (APHA, 2005).
205
206 *2.5. Statistical analysis*
207 The mean and standard deviations were calculated for duplicate bottles. Statistical
208 analysis was performed by analysis of variance (ANOVA), followed by Tukey tests to
209 identify the significance of the data obtained using Statgraphics Centurion software
210 version 18. Comparisons with a value of $p < 0.05$ were considered significant.
211
212 **3. Results and Discussion**
213 *3.1. Influence of the PWW load*
214 PPB were only able to grow in 5, 10 and 15 fold diluted piggery wastewaters as shown
215 by the increase in OD_{808} over the 20 days of experiment (Fig. 1A). Biomass growth
216 mainly occurred during the first 10 days. A limited PPB growth was observed in
217 undiluted PWW likely due to the high NH_4^+ concentrations present in the PWW (Getha

et al., 1998) and to the fact that PPBs are not able to compete with chemotrophic bacteria as a result of the limited availability of light (Siefert et al., 1978). Indeed, photosynthesis and consequently the production of ATP for the photoassimilation of organic/inorganic compounds, are negatively affected in high turbidity media. The control tests (non-inoculated 10 fold diluted PWW) did not experience any significant increase in OD_{808} along the 20 days of experiment. Similarly, an increase in biomass concentrations (estimated by the difference between the final and initial TSS in the cultivation broth) of 0.72 ± 0.17, 0.74 ± 0.08 and 0.57 ± 0.04 g TSS L^{-1} was recorded in 5, 10 and 15 fold diluted PWW, respectively, compared to 0.21 ± 0.04 and -0.03 ± 0.01 g TSS L^{-1} in the undiluted PWW and control tests (Fig. 2A). The lower biomass concentration recorded in 15 fold diluted PWW was likely due to the lower concentration of biodegradable carbon and the high pH value in the cultivation broth of this test (as latter described in the sections below).

<Figure 1>

<Figure 2>

TOC removal efficiencies (REs) of 25 ± 4%, 29 ± 1% and 34 ± 3% were achieved in 5, 10 and 15 fold diluted tests, respectively, after 20 days of experiment (Fig. 1B). Organic carbon removal was correlated with biomass growth, and mainly occurred during the first 10 days, which suggests the assimilatory nature of TOC removal mechanisms. The rapid decrease in TOC concentration observed in tests with raw PWW (absence of significant biomass growth) and 5 fold diluted PWW during the first 6 days (Fig. 1B) was attributed to an experimental error during the first three samplings, where part of the organic matter pelletized after centrifugation was resuspended. Negligible variations in TOC concentrations of 5.8 ± 0.4% were recorded in the control test. PPBs encode a large number of metabolic pathways and can degrade multiple carbon sources such as

small molecules of fatty acids, alcohols, carbohydrates and a limited number of amino acids (Hülsen et al., 2018a; Lu et al., 2019b). More specifically, *Rhodopseudomonas palustris* has the complete tricarboxylic acid cycle (TCA), the Embden-Meyerhof pathway and the pentose phosphate pathway (Larimer et al., 2004). Under anaerobic conditions and infrared radiation supply, the anoxygenic photosynthesis would generate the energy necessary for the degradation of organic pollutants. This explains the rapid assimilation of TOC observed during the first days of the experiment.

Final TN removals of 14 ± 5%, 18 ± 2% and 21 ± 1% were recorded in 5, 10 and 15 fold diluted tests, respectively. These removals were also correlated with biomass growth and TOC removal, which confirms the assimilatory nature of the nitrogen removal mechanisms. In this context, PPBs are capable of assimilating all forms of nitrogen (NO_3^-, NO_2^-, NH_4^+, N_2 and organic N), with confer these microorganisms a high potential for wastewater bioremediation (Lu et al., 2019b). The elemental composition of PPBs cells in terms of C, N, H and O typically accounts for 52%, 11%, 8% and 29%, respectively, with a carbon:nitrogen (C:N) ratio of 5:1 (Carlozzi et al., 2006). In this context, the ratio of C:N removed from PWW was 5:1 in PPB tests conducted with 10 or 15 folds diluted PWW (478 mg C removed:95 mg N removed; 381 mg C removed:75 mg N removed). Therefore, all C and N removed was used for microbial growth (assimilative removal). Negligible nitrogen removal efficiencies (~ 1% TN REs) were observed in the control test in the absence of PPB. On other hand, the pH increased from 8.00 at the beginning of the tests, up to 8.55 ± 0.13, 8.84 ± 0.06 and 8.92 ± 0.14 in the 5, 10 and 15 fold diluted tests, and remained stable in undiluted and control tests (Fig. S3A). This increase in the pH of the culture broth was likely due to the consumption of organic acids and CO_2 by PPB (Hülsen et al., 2014).

CO_2 concentration decreased in the headspace of the batch photobioreactors where PPB growth occurred. Thus, a decrease in CO_2 concentrations from 31.4 ± 3.2 to 14.6 ± 5.2 g m^{-3} was recorded in 5 fold diluted tests, while an almost complete CO_2 depletion occurred in the headspace of the tests conducted with 10 and 15 fold diluted PWW (Fig. S4A1). This can be explained by anaerobic carbon fixation by PPB in the presence of infrared light and by the increase in the pH of the cultivation broth (which mediated CO_2 absorption by the culture medium). CO_2 concentration in the headspace of undiluted PWW tests increased from 113 ± 1 to 241 ± 13 g m^{-3} and from 20.5 ± 0.8 to 30.8 ± 1.2 g m^{-3} in the control test, as a result of organic matter oxidation. On the other hand, H_2S concentration only increased in undiluted and 5 fold diluted tests up to 7.1 ± 2.6 and 1.5 ± 0.4 g m^{-3}, respectively, which was likely due to sulphate reduction during anaerobic TOC oxidation. Finally, CH_4 concentration increased in undiluted, 5 and 10 fold diluted tests up to 1.3 ± 0.0, 11.5 ± 0.2 and 4.0 ± 1.9 g m^{-3}. Interestingly, the higher concentration of CH_4 (produced from the anaerobic digestion of TOC) was recorded in 5 fold diluted PWW, where methanogenesis was not likely inhibited by the high NH_4^+ concentrations present in raw PWW (Nakakubo et al., 2008; Yenigün and Demirel, 2013). A gradual development of anaerobic communities in 5 fold diluted tests was likely to occur since genes related to classical metabolic pathways for the generation of CH_4 and H_2S in *R. palustris* have not been described in literature (Larimer et al., 2004).

The results indicate that at a higher dilution of PWW, the removal of carbon and nitrogen was favored, mainly due to the increase in light penetration in the systems and to the decrease in the inhibition effect by high concentrations of NH_4^+. Ten fold diluted PWW was selected for further experiments based on the similar biomass production compared to 5 fold dilution, the absence of H_2S generation, along with TOC and TN removals comparable to those achieved in 15 fold diluted tests.

292

293 *3.2. Influence of O_2 on PPB growth and nutrient recovery*

294 PPB were able to grow regardless of the extent of air supply as shown by the increase in
295 OD_{808} (Fig. 3A). However, a decrease in the absorbance in the tests inoculated with
296 PPB under an open atmosphere was observed from the day 8 onward. This decrease was
297 due to the depletion of the carbon source as described below. Thus, an increase in TSS
298 concentration (estimated as the difference between the final and initial biomass
299 concentrations) of 0.43 ± 0.01 g L^{-1} was recorded in aerated PPB tests, which was
300 significantly lower than the biomass production at the end of the tests with PPB under a
301 He atmosphere (1.31 ± 0.07 g L^{-1}) and with PPB periodically supplied with 20 mL of air
302 (1.01 ± 0.01 g L^{-1}) (Fig. 2B). These results confirm that PPBs can grow under aerobic
303 conditions, although PPB growth is favored under anaerobic conditions in the presence
304 of infrared radiation. Hence, phototrophic growth is favored under anaerobic conditions,
305 in the absence of the inhibition of bacteriochlorophyll synthesis induced by oxygen but
306 under chemotrophic growth. Finally, OD_{808} remained constant over time in the control
307 tests, although PPB may naturally acclimate and grow under longer periods of time due
308 to the intrinsic presence of these bacteria in wastewaters (García et al., 2019; Hülsen et
309 al., 2014; Siefert et al., 1978).

310 <Figure 3>

311 TOC-REs of 37 ± 3% and 41 ± 2% were obtained in tests inoculated with PPB
312 incubated under a He atmosphere and with periodic air supplementation, respectively
313 (Fig. 3B). The slightly higher TOC-REs in the presence of O_2 suggests that the
314 contribution of the oxidative phosphorylation of PPB is promoted under micro-aerobic
315 conditions (Lu et al., 2011; Meng et al., 2017). PWW treatment under an open

316 atmosphere resulted in an increase in TOC-REs up to 83 ± 2% and 83 ± 3% in tests
317 inoculated with PPB and without inoculation, respectively, although TOC removal was
318 initially faster in tests inoculated with PPB, which are known to aerobically assimilate
319 organic matter. On the other hand, the degradation of VFAs was correlated to TOC
320 removal (Fig. 3C, plotted as the carbon contained in all VFAs). Indeed, VFA-REs of 47
321 ± 15% and 45 ± 11% were recorded in PPB tests conducted under a He atmosphere
322 without and with addition of air, respectively. This partial assimilation of VFAs was
323 likely due to both the lack of electron acceptor in the cultivation broth and the inhibition
324 mediated by the increase in pH. A negligible degradation of VFAs was recorded in the
325 control tests, while high VFA-REs of 83 ± 1% and 90 ± 7% were achieved in the open
326 photobioreactors with and without PPB inoculation, respectively. Interestingly, the high
327 TOC and VFA removals in the non-inoculated aerobic PWW biodegradation tests
328 entailed a decrease in the final TSS concentration (Fig. 2), which confirmed the absence
329 of growth of chemotrophic bacteria (described as bacteria with an efficient fermentative
330 energy metabolism) (Siefert et al., 1978) and which suggests that VFAs were stripped
331 out in the open photobioreactors. VFA depletion by day 8 in open photobioreactors
332 inoculated with PPB correlated with the decline in OD_{808}, which confirmed that VFAs
333 were the main substrate of PPB. Interestingly, the occurrence of aerobic conditions
334 during PWW degradation did not increase PPB growth, but resulted in significant
335 carbon losses. Hence 133 ± 10%, 87 ± 5% and 19 ± 0% of the carbon removed was
336 recovered in the form of biomass in the tests conducted with PPB under a He
337 atmosphere, with periodic air supplementation and under an open atmosphere,
338 respectively. The high carbon recovery under a He atmosphere was likely due to an
339 experimental error in the determination of biomass concentration. VFAs mixtures
340 support superior PPB growth rates compared to individual VFA solutions, propionic

acid being the preferred VFA by PPB (Alloul et al., 2019). Indeed, propionic acid was completely consumed in all tests inoculated with PPB. Moreover, VFAs can be metabolized by VFA catabolic pathways and converted into Acetyl-CoA for subsequent degradation in the TCA cycle in PPB.

TN-REs of 29 ± 3%, 33 ± 1%, 32 ± 1% and 12 ± 1% were recorded in tests inoculated with PPB under a He atmosphere, with periodic air dosing, under an open atmosphere and in non-inoculated tests in open photobioreactors, respectively (Fig. 3D). A low TN-REs of 4.8 ± 0.1% was recorded in the control test with PWW under a He atmosphere. The absence of biomass growth in non-inoculated tests in open photobioreactors suggests that TN removal was due to NH_4^+ volatilization, while nitrogen assimilation into biomass (i.e. protein formation) was the main mechanism in enclosed photobioreactors. Overall the TN recovered in the form of biomass decreased from 101 ± 13% to 64 ± 0% and 26 ± 1% under periodic O_2 dosing or open photobioreactors. PPB can support an efficient assimilation of TN in domestic wastewater treatment (Hülsen et al., 2014), with 99.6% N removal efficiencies under anaerobic conditions and infrared radiation, but domestic wastewater presents lower concentrations of nitrogen (46 mg NH_4-N L^{-1}) compared to 10 fold diluted PWW (503 ± 33.9 mg N L^{-1}). Therefore, this study showed that PPBs can grow at high nitrogen concentrations, and agree with literature studies that reported growth of pure cultures of PPB at 650 mg NH_4Cl L^{-1} (Carlozzi and Sacchi, 2001) or up to 8000 mg N L^{-1} (Meng et al., 2018). Unfortunately, only the final pHs were recorded in this test due to a failure in the pH electrode. Thus, pH values of 7.89 ± 0.02, 9.51 ± 0.08 and 9.38 ± 0.07 were recorded in the control test, in the test inoculated with PPB under a He atmosphere and with periodic dosing of air, respectively, while pH of 9.03 ± 0.09 and 8.93 ± 0.20 were recorded in the open tests with and without PPB inoculation, respectively.

CO_2 concentration in the headspace of the photobioreactors was depleted in all closed tests except the control, where an increase from 12.2 ± 0.3 to 26.7 ± 1.5 g m^{-3} was observed (Fig. S4A2). Finally, the presence of H_2S and CH_4 was not detected in the headspace of the photobioreactors during the course of this experiment (Fig. S4B2, S4C2).

PPBs are photoheterotrophs and the presence of oxygen in the cultivation broth is known to inhibit the synthesis of bacteriochlorophyll (Izu et al., 2001). However, recent studies have reported that microaerophilic conditions during PPB cultivation can substantially improve the efficiency of PPB-based wastewater treatment in photobioreactors operated with mixed cultures (Lu et al., 2019a; Peng et al., 2018; Yang et al., 2018). Under microaerophilic conditions it is very likely that aerobic organisms co-exist together with PPBs, which allows to anticipate that a symbiosis between PPBs and heterotrophic aerobes can be beneficial in photobioreactors devoted to wastewater treatment, provided that the Redox potential is maintained in negative values (Siefert et al., 1978).

In brief, PPBs were able to assimilate carbon/nutrients and grow both anaerobically and aerobically, which confirms their high metabolic plasticity. A photoautotrophic metabolism based on the fixation of CO_2 and energy obtained from photosynthesis was initially observed, with a gradual contribution of a photoheterotrophic metabolism in the tests under a Helium atmosphere. Under an air atmosphere, a large fraction of the organic matter was removed via volatilization since the amount of biomass produced did not correlate with TOC or VFA degradation. In PPB-Air, the high organic matter content at the early stages of biodegradation favored anaerobic conditions and promoted PPB growth (as indicated by the increase in OD_{808}), while bacteriochlorophyll synthesis

was inhibited in the presence of oxygen at the latest stages of the test, when VFA were depleted.

The feasibility of scaling wastewater treatment with PPB in photoanaerobic systems is under investigation at semi-industrial scale within the first photobiorefinery in Europe, constructed in the framework of the BBI-H2020 Deep Purple project focused on the extraction and recovery of high value-added resources with PPB (https://deep-purple.eu/).

3.3. Influence of CO_2 and $NaHCO_3$ on PPB growth and nutrient recovery

The growth of PPB was significantly favored by the addition of CO_2 to the photobioreactor headspace (Fig. 4A). Thus, the OD_{808} in the test inoculated with PPB and supplemented with CO_2 increased by 80% compared to the tests without CO_2 addition. Interestingly, $NaHCO_3$ addition did not result in a significant increase or decrease in PPB growth. The increase in TSS concentrations (estimated as the difference between the final and initial biomass concentrations) accounted for 1.00 ± 0.06, 1.81 ± 0.07 and 1.09 ± 0.13 g L^{-1} for PPB, PPB with CO_2 addition and PPB with $NaHCO_3$ addition, respectively, which confirmed the beneficial effect of CO_2 addition (Fig. 2C). The supplementation of trace elements by day 22 did not result in an enhancement of PPB growth and revealed that PWW biodegradation was not limited by essential micronutrients.

<Figure 4>

Removal efficiencies of TOC of 31 ± 0% and 36 ± 1% were achieved in tests with PPB and PPB with $NaHCO_3$ addition, respectively (Fig. 4B). A significant increase in TOC RE up to 72 ± 3% was recorded when PPB were supplemented with CO_2, which was

414 initially attributed to the beneficial effect of CO_2 as electron acceptor. The enclosed
415 nature of the experimental set-up entails that TOC removal was caused by carbon
416 assimilation into PPB biomass. No significant variation in TOC concentration
417 throughout the experiment was observed in the control tests. Similarly, VFA-REs of 26
418 ± 3%, 74 ± 13% and 26 ± 16% were achieved in tests with PPB, PPB with CO_2 addition
419 and PPB with $NaHCO_3$ addition, respectively (Fig. 4C). The lag phase in VFA
420 consumption initially observed in Figure 4C was due to the preferential assimilation of
421 highly reduced soluble organic compounds (not determined in this study) present in
422 PWW. On the other hand, negligible variations in VFA concentration were recorded in
423 the control tests, which agreed with TOC measurements.

424 Final TN removals of 15 ± 2%, 33 ± 3% and 21 ± 1% were recorded in tests with PPB,
425 PPB with CO_2 addition and PPB with $NaHCO_3$ addition, respectively. The superior
426 removal of TN mediated by CO_2 supplementation correlated with the growth of PPB
427 and TOC removal, and points out toward assimilation as the main nitrogen removal
428 mechanisms. On other hand, pH values increased from 8.08 ± 0.07 to 8.80 ± 0.03 and
429 8.91 ± 0.15 in tests with PPB and PPB with $NaHCO_3$ addition, respectively (Fig. S3B),
430 while CO_2 addition maintained the pH stable at 8.12 ± 0.00 (as a result of the
431 acidification caused of this gas), values similar to those recorded in the control test
432 (8.18 ± 0.00).

433 All CO_2 concentration initially present in the headspace of the photobioreactors was
434 absorbed in the tests with PPB and PPB with $NaHCO_3$ addition (1.7 ± 0.2 g m^{-3}) despite
435 TOC mineralization, which highlights the key role of the pH increase in CO_2
436 sequestration. The concentration of CO_2 in the PPB test supplemented with CO_2
437 remained stable for the first 8 days (when pH increased) and increased approximately
438 by 34.4 ± 11.8 g m^{-3} every two days afterwards (Fig. S4A3). An increase in CO_2

concentration up to 41.4 ± 1.5 g m^{-3} was recorded in the control tests concomitantly with an increase in H_2S concentration up to 4.49 ± 0.16 g m^{-3} from day 14, which was mediated by sulphate reducing bacteria oxidizing a fraction of the VFAs. H_2S and CH_4 were not detected in the headspace of the photobioreactors inoculated with PPB.

A sequence of photoheterotrophic metabolic routes was hypothesized in test performed with PPB and CO_2 addition, where photoheterotrophy of non-VFA reduced organic substrates occurred in the first 8 days. This was evidenced by the consumption of a fraction of TOC, while VFA concentrations remained constant. In addition, an intensive usage of CO_2 occurred in the initial stages of PWW biodegradation to support reduced organics assimilation by directing the excess of reductive power through the Calvin Cycle (McKinlay and Harwood, 2010). After 8 days, the phototrophic consortia started to assimilate VFAs at high uptake rates (Fig. 4C), concomitantly with an increase in CO_2 headspace concentration without a significant increase in the pH of the cultivation broth. This confirmed the beneficial role of CO_2 addition on PPB mediated PWW treatment.

3.4. Influence of pH on PPB growth and nutrient recovery

PPB growth in tests supplemented with CO_2 and with pH control at 7 was very similar (Fig. 5A). The OD_{808} of the PPB culture without pH control or CO_2 addition increased up 1.25, values similar to those obtained in previous tests. Likewise, the control tests did not experience any significant increase in OD_{808} along the 34 days of experiment. Final biomass productions (estimated as the difference between the final and initial TSS) of 0.83 ± 0.21, 1.65 ± 0.30, 1.58 ± 0.04 and 2.00 ± 0.14 g TSS L^{-1} were recorded in tests with PPB, PPB with CO_2 addition, PPB with pH controlled to maintain similar

values to the test with CO_2 addition and PPB with pH control at 7, respectively. These results clearly demonstrated the prominent role of pH on PPB growth under these experimental conditions.

<Figure 5>

TOC removal efficiencies of 30 ± 1%, 69 ± 3%, 58 ± 2% and 75 ± 2% were achieved in tests with PPB, PPB with addition of CO_2, PPB with pH controlled to maintain similar values to the test with CO_2 addition and PPB with pH control at 7, respectively. Similarly, the degradation of VFAs also occurred with efficiencies 49 ± 1%, 92 ± 3%, 77 ± 1% and 98 ± 1%. The highest removals of TOC and VFA were obtained in the PPB tests with pH maintained at 7. These removals were significantly higher than those recorded in the tests with addition of CO_2 and with pH controlled to maintain similar values, thus confirming the key role of pH on organic matter removal by PPB during PWW treatment. VFAs are the main constituent of PWW organic matter, representing 80% of the carbon present in this type of wastewater (1.2 gVFAs (C) L^{-1} versus 1.5 gTOC L^{-1}). It has been consistently described in literature that PPBs are capable of assimilating a large number of VFAs as a carbon source (Wei et al., 2016), while some species such as *Rhodopseudomonas* have the ability to use all short-chain VFAs (C2-C6) in comparison to *Rhodobacter* (Okubo et al., 2005). A large concentration of VFAs can result in inhibition of PPB growth (Ghosh et al., 2017), while the consumption of VFAs mediates an increase in pH due to the acidic nature of these compounds, which might ultimately inhibit PPB growth.

Final TN removals of 25 ± 3%, 38 ± 4%, 34 ± 4% and 39 ± 3% were recorded in tests with PPB, PPB with addition of CO_2, PPB with pH controlled to maintain similar values to the test with CO_2 addition and PPB with pH control at 7, respectively. Negligible variations in TN concentration were recorded in the control test with PWW under a He

atmosphere. The highest TN removal was obtained in the PPB test with pH adjusted to 7, condition supporting also the highest biomass production and TOC removal. The values of pH increased from 7.96 ± 0.02 to 8.87 ± 0.27 in the PPB test without pH control or CO_2 addition, and from 7.46 ± 0.02 to 7.94 ± 0.04 in PPB tests with addition of CO_2. The benefits derived from CO_2 addition were induced by the indirect pH control exerted in the cultivation broth, which showed an optimum performance at pH 7. The optimal pH range described for *R. palustris* is 6-8.5 (van Niel, 1944), with bacterial growth inhibition occurring at higher pH values. PPB inhibition at high pH values was likely due to the loss of the electrochemical potential between the cultivation medium and the cytoplasm, which decreases the proton motive force necessary for the synthesis of ATP by ATP-synthase during photosynthesis.

The CO_2 concentration present in the photobioreactor headspace was absorbed in the tests with PPB (Fig. S4A4). No significant variations were observed in the tests with PPB with pH controlled to maintain similar values to the test with CO_2 addition and control test, where CO_2 concentrations remained constant at 10.9 ± 4.2 and 23.9 ± 2.5 g m^{-3}, respectively. An increase in CO_2 concentration was recorded in the test with CO_2 addition from day 10 onwards and in the tests with pH 7 from day 20 onwards. The increase in the concentration of CO_2 observed in the test with pH control at 7, was likely due to the swift in the metabolism of PPB form a carbon assimilatory to a dissimilatory metabolism. Neither H_2S nor CH_4 were detected in the headspace of the photobioreactors during the course of this experiment regardless of the conditions tested.

In brief, phototrophic metabolism was highly efficient for the treatment of PWW under neutral pH. Similarly, empirically validated simulations of *R. palustris* metabolism under anaerobic conditions with acetate in minimal media resulted in an increase in pH,

513 which confirms that proton metabolism plays a key role for optimal growth in PPB
514 (Navid et al., 2019).

515

516 **4. Conclusions**

517 PWW treatment using mixed cultures of PPB under infrared radiation represents a
518 promising platform for resource recovery under optimized operational conditions.
519 PWW dilution is required to prevent the inhibition of PPB as a result of the high
520 strength of this wastewater. Resource recovery using PPB seems to be hindered by the
521 presence of air. CO_2 addition was identified as an effective operational strategy to
522 maximize carbon and nitrogen removal from PWW along with PPB growth. However,
523 the beneficial effects from CO_2 supplementation derived from pH control, which is the
524 actual key control parameter on PPB-based PWW valorization.

525

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531 technical assistance in the TOC/VFAs analysis and calibration of materials used,
532 respectively.

533

534 **Supplementary Materials**: Figure S1: Absorption spectrum (350-850 nm) of PWW
535 and PPB in 10 fold diluted PWW at the beginning and end of the assay (Test series 1).
536 Figure S2: Photograph of the experimental set-up with photobioreactors with PWW
537 diluted 10 fold (left) and bottles inoculated with PPB in 10 fold diluted PWW (right)

under a He atmosphere. Figure S3: Trend of pH during PWW biodegradation in Test series 1 (A), Test series 3 (B) and Test series 4 (C). Figure S4: Trend of the gas concentration of CO_2 (A), H_2S (B) and CH_4 (C) in the headspace of the photobioreactors in Test series 1 (1), Test series 2 (2), Test series 3 (3) and Test series 4 (4).

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706 **List of figures**

707

708 **Figure 1.** Trend of culture absorbance (A), and TOC (B) and TN (C) concentrations
709 during piggery wastewater biodegradation by PPB in raw PWW (▲) (secondary axis
710 values in gray), 5 times diluted PWW (◊), 10 times diluted PWW (■), 15 times diluted
711 PWW (○) and non inoculated PWW (∗).

712

713 **Figure 2.** Variation in TSS concentration during piggery wastewater biodegradation in
714 test 1 (A), test 2 (B), test 3 (C) and test 4 (D). Values represented the difference between
715 the final and initial TSS concentrations.

716

717 **Figure 3.** Trend of culture absorbance (A), and TOC (B), volatile fatty acid (C) and TN
718 (D) concentrations during 10 times diluted piggery wastewater biodegradation by PPB
719 (■), PPB with air dosing (△) and non-inoculated PWW (∗) in closed photobioreactors,
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721 photobioreactors.

722

723 **Figure 4.** Trend of culture absorbance (A), and TOC (B), volatile fatty acids (C) and
724 TN (D) concentrations during 10 times diluted piggery wastewater biodegradation by
725 PPB (■), PPB with CO_2 supplementation (▲), PPB with $NaHCO_3$ supplementation (◊)
726 and non-inoculated PWW (∗).

727

728 **Figure 5.** Trend of culture absorbance (A), and TOC (B), volatile fatty acids (C) and
729 TN (D) concentration during 10 times diluted piggery wastewater biodegradation by
730 PPB (■), PPB with CO_2 supplementation (△), PPB with pH controlled to maintain
731 similar values to the test with CO_2 addition (◊), PPB with pH control at 7 via HCl
732 addition (●) and non-inoculated PWW (∗).

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747 **Figure 1.**

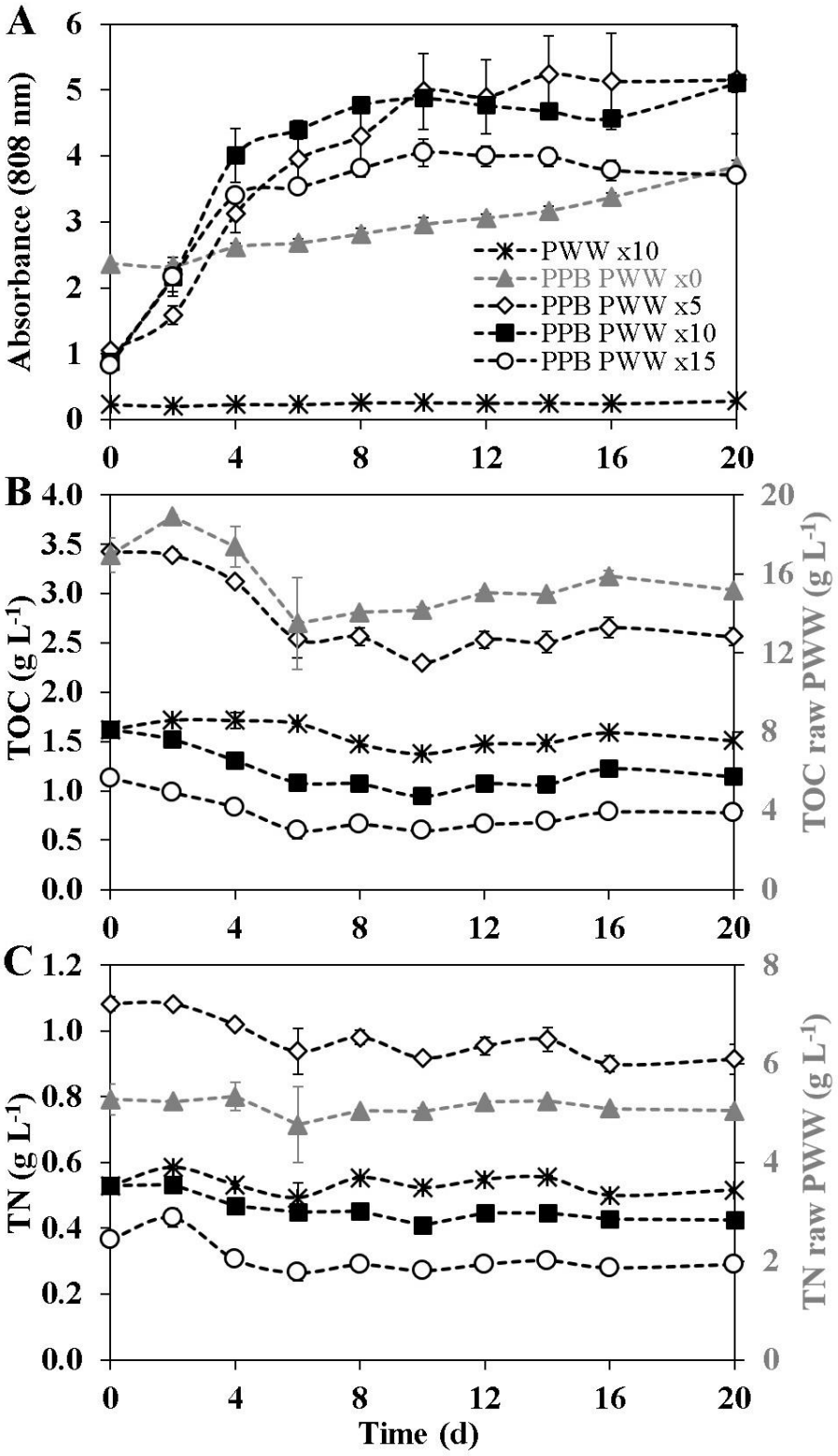

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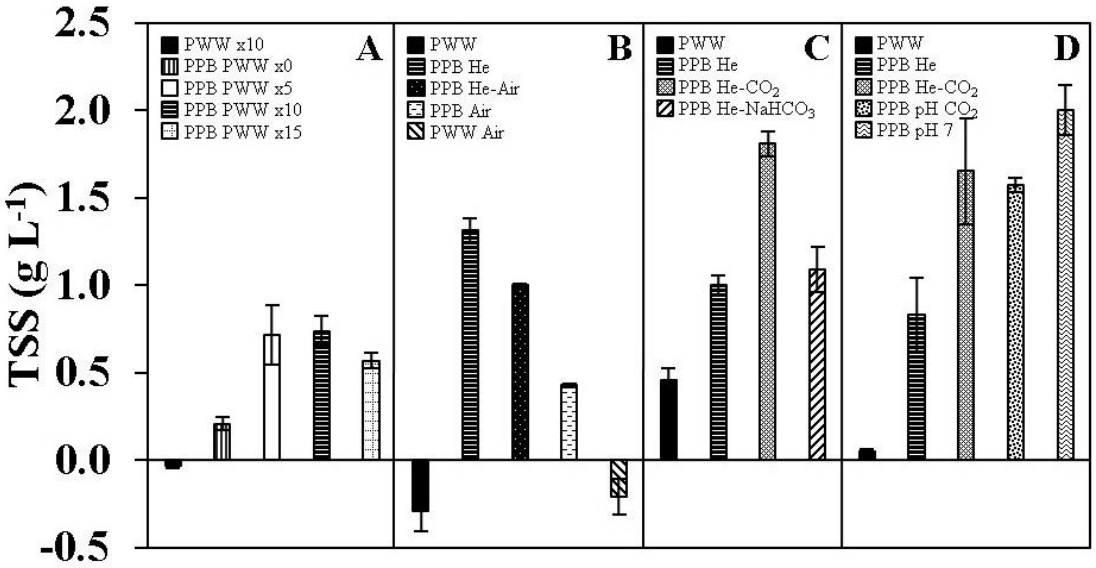
Figure 2.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2.0
1.5
1.0
0.5
0.0
-0.5
A
PWW x10
PPB PWW x0
PPB PWW x5
PPB PWW x10
PPB PWW x15
B
PWW
PPB He
PPB He-Air
PPB Air
PWW Air
C
PWW
PPB He
PPB He-CO2
PPB He-NaHCO3
D
PWW
PPB He
PPB He-CO2
PPB pH CO2
PPB pH 7

764 **Figure 3.**

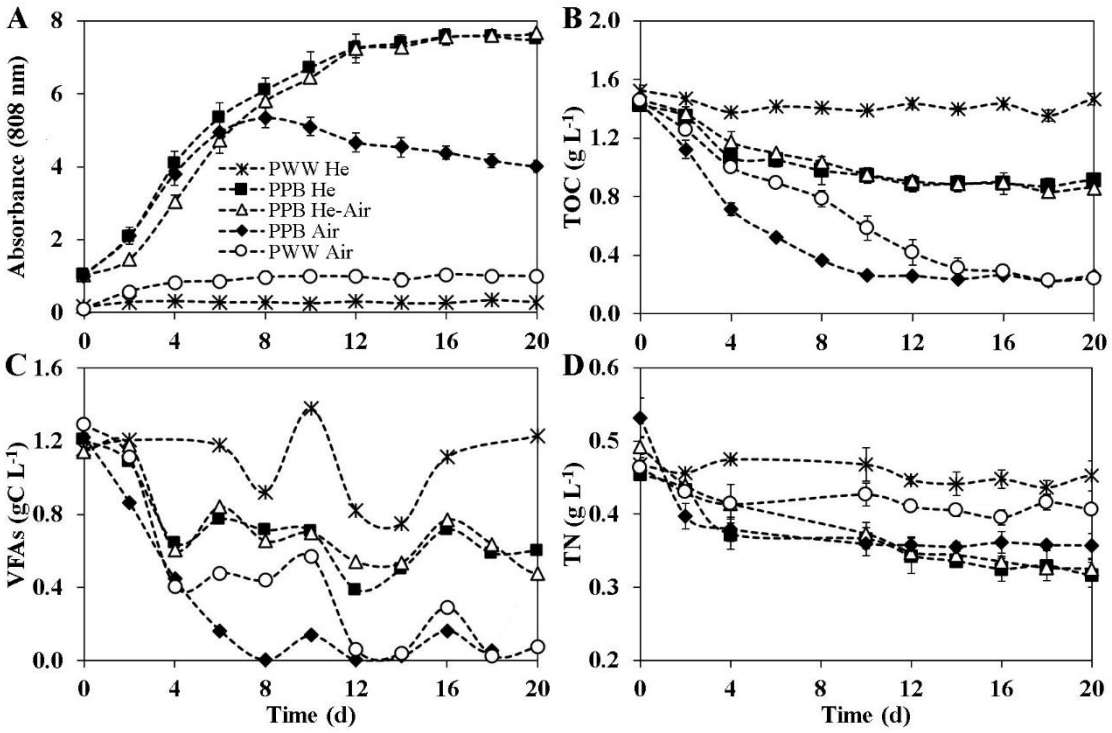

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Figure 4.

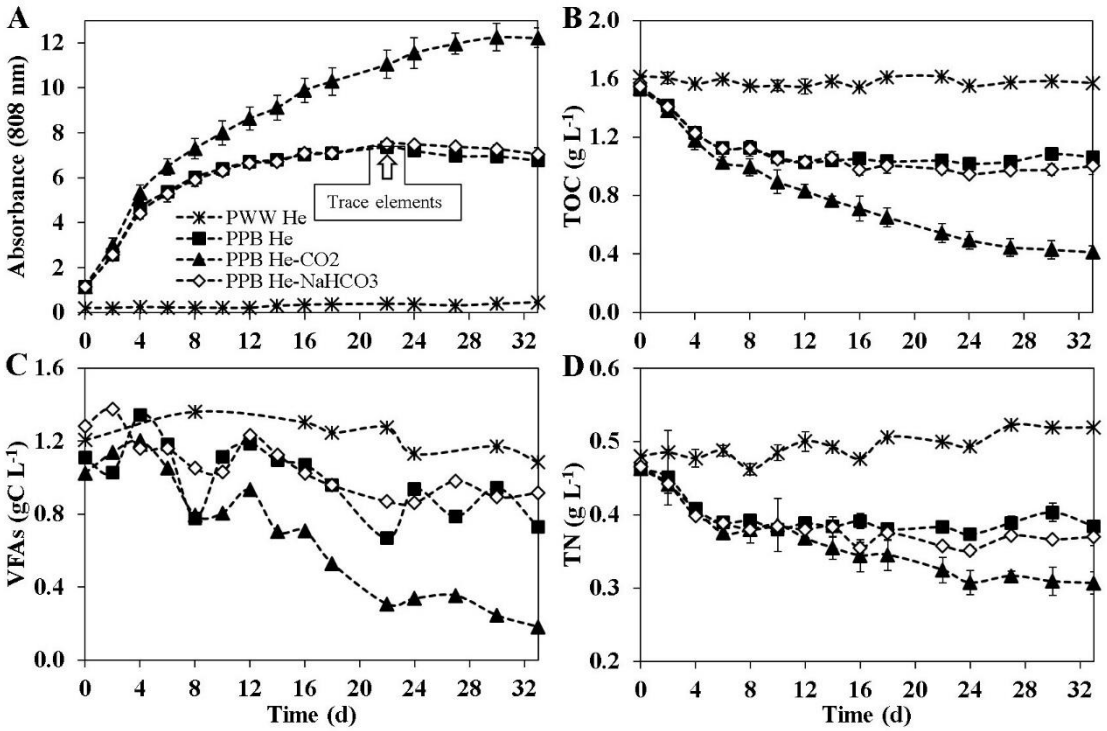
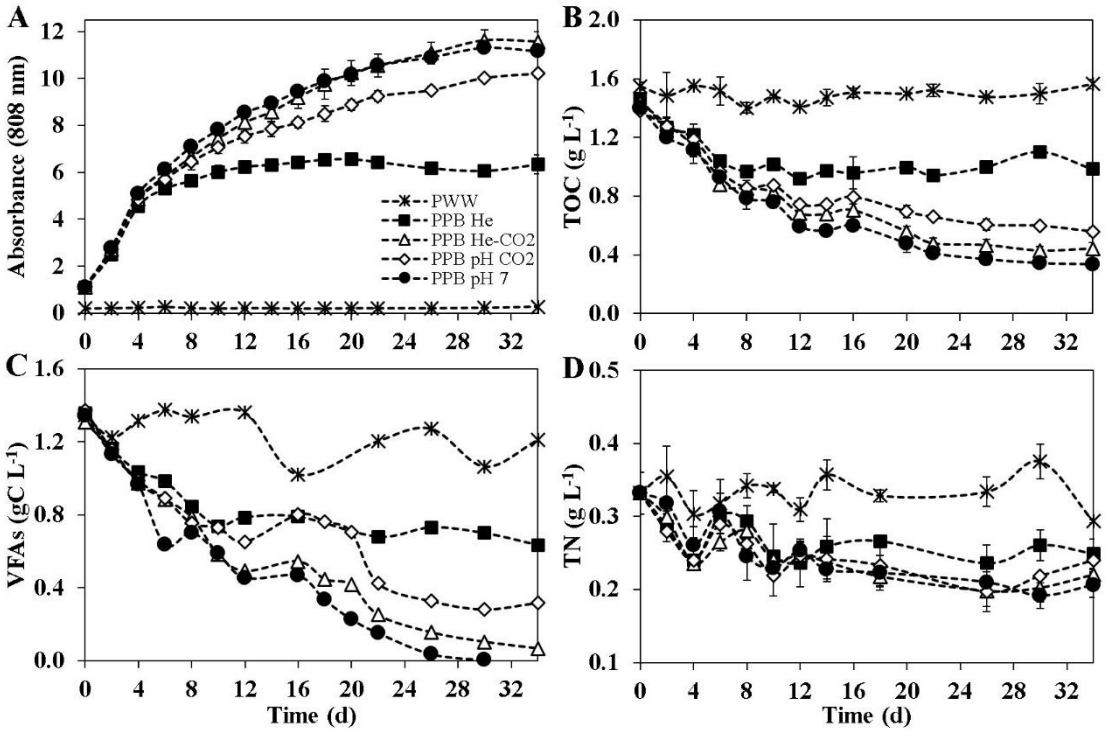

A
Absorbance (808 nm)
Trace elements
PWW He
PPB He
PPB He-CO2
PPB He-NaHCO3
B
TOC (g L-1)
C
VFAs (gC L-1)
Time (d)
D
TN (g L-1)
Time (d)

Figure 5.


A
Absorbance (808 nm)
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PPB He
PPB He-CO2
PPB pH CO2
PPB pH 7
B
TOC (g L-1)
C
VFAs (gC L-1)
D
TN (g L-1)
Time (d)
Time (d)