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Effect of organic carbon enrichment on the treatment efficiency of primary settled wastewater by *Chlorella vulgaris*

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Abstract

This work evaluated the performance of a microalgae treatment process for settled municipal wastewater in a laboratory setting under static culturing conditions, as an alternative to traditional, energy intensive secondary and tertiary wastewater treatment systems. Primary tank settled wastewater (PSW) was first enriched with small quantities of glucose (<300 mg L^{-1}) as an organic carbon source to facilitate the bioremediation by the mixotrophic microalga *Chlorella vulgaris*. Characterisation of the wastewater revealed significant reductions in NH_3-N (from 28.9 to 0.1 mg L^{-1}) and PO_4-P, (from 3.2 to 0.1 mg L^{-1}) in just 2 days. Additionally, the exogenous glucose appeared completely removed from the wastewater after the first day. These achieved levels of treatment in respect of both the NH_3-N and PO_4-P were much higher than those recorded without *C. vulgaris* treatment with or without glucose enrichment. This would mean that the microalgae were chiefly responsible for removing the inorganic nitrogen and phosphorus, while the naturally occurring heterotrophic organisms had consumed the carbonaceous matter. The reliability of this process was evaluated across a further three independent batches of PSW with varying compositions of these inorganics and chemical oxygen demand using alternative organic (glycerol) and inorganic (CO_2) carbon sources. The efficiency of the microalgae treatment process at reducing NH_3-N and PO_4-P was consistent in PSW enriched with organic carbon, resulting in >90% reduction of the inorganic compounds in each batch. The results demonstrate that microalgal culturing processes to treat PSW in bioreactors without aeration are a key area to develop as an alternative biological treatment option.

2

1. Introduction

Wastewater treatment is necessary to limit the potential impacts of pollution and eutrophication on receiving aquatic systems. Its main aim is towards the significant reduction of carbonaceous (organic) materials and, where sensitive surface waters are involved, nutrients (i.e. phosphorus (P) and nitrogen (N) compounds). The main phase of wastewater treatment is biological, essentially performed by microorganisms, such as in the activated sludge process or the biological nutrient removal process; these processes are conventionally termed the secondary treatment phase [1]. These secondary treatment processes are dependent on oxygen (O_2) to enable the endogenous microorganisms present to breakdown and assimilate the organic and inorganic matter. This stipulation for O_2 comes at a high cost with wastewater treatment consuming approximately 1 to 3% of the total electricity generated in developed nations of which 40 to 60% is expended on supplying air to the aeration basin [2–4]. This is important considering the cost to treat wastewater is projected to rise as a result of growing urbanisation and the proposition of more stringent effluent requirements. For example, the enactment of the Urban Wastewater Treatment Directive sets European discharge limits at 2 or 1 mg L^{-1} total phosphorus (TP) for population equivalent of <100k or >100k, respectively [5]. These discharge limits contribute considerably to the natural P concentrations in riverine and estuarine environments [6], and decreasing inputs of P to receiving systems is considered key to reducing eutrophication [7]. In order to limit phytoplankton growth and thus eutrophication in receiving waters, discharge TP concentrations of <0.5 mg L^{-1} is necessary and currently under consideration [8].

In recent decades, policies to safeguard water resources have influenced the development of wastewater treatment systems and its management, including a focus on

72   energy consumption and the sustainable performance of these industrial processes. Given

73   the importance of wastewater treatment, a key question is how to reduce energy

74   consumption of this process without affecting performance in respect to meeting water

75   discharge limits. One direction towards making wastewater treatment more sustainable is

76   to recover the resources that it holds, such as water, nutrients (e.g. P and N) and energy.

77   Verstraete *et al*., (2009) estimated the total value of resources which could be recovered

78   from wastewater at € 0.35 per m^3 based on 2009 market prices. The shift of wastewater

79   treatment from being an end-of-pipeline process to a resource has seen the development

80   and operation of technologies such as sludge digestion for methane production, the

81   integration of energy capturing technology utilising the wastewater treatment

82   infrastructure and nutrient recovery aimed at P and N [2,10].

83   One particular option for the remediation and capture of inorganic N and P from

84   wastewater is using microalgae. The rationale behind this approach lies in the ability of

85   mixotrophic microalgae to utilise organic and inorganic carbon, as well as the N and P in

86   wastewater for their growth, hence leading to a reduction in the concentration of these

87   substances that will meet discharge limits. Simultaneously, energy-rich microalgal biomass

88   is produced that could be recovered and utilised for the generation of energy or other

89   products following further processing. The remediation potential of this approach has been

90   evaluated for use in an array of wastewater types with promising results [11,12]. A further

91   benefit of microalgae incorporation into wastewater treatment is their generation of

92   dissolved O_2 via photosynthesis. Photosynthetic oxygenation has the potential to meet

93   dissolved O_2 needs to a treatment system without the use of mechanical aeration or mixing,

94   thereby reducing the energy demand for the treatment process. To exemplify, Karya *et al*.

95   (2013) employed a sequence batch design with *Scenedesmus* sp. and nitrifying bacteria

96   isolated from activated sludge to evaluate whether this co-culture system can support

97   nitrification. Without mechanical aeration, the process was shown successful in reducing 81

98   to 85% of ammonium-nitrogen through its conversion to nitrate-nitrogen by nitrification, for

99   which the O_2 for this process had been generated by the microalga. Further support for a

100 microalgae-based wastewater treatment approach as a viable biological system relates to its

101 general improved performance in the presence of bacteria.   Although considered

102 unavoidable and a major challenge because of the potential to out-compete algae, the

103 presence of bacteria in co-culture with mixotrophic microalgae has been shown to respond

104 better in treating wastewater compared to the use of axenic cultures [14,15]. This affect

105 has been attributed to the exchange of co-factors between the microalgae and bacteria,

106 which include growth promoting compounds and vitamins [16].   Furthermore, when

107 compared to current secondary treatment systems, microalgae also provide a potential

108 system for sequestering carbon as well as removal of micro-pollutants and toxic metals [17].

109   Despite these advantages, there are various practical and economical challenges that

110 still limit the implementation of microalgae-bacteria co-cultures for wastewater treatment.

111 One such challenge is the cultivation process. As with most conventional wastewater

112 treatment operations, aeration systems are used in microalgae culturing to provide mixing

113 for improving the exchange of O_2 and carbon dioxide (CO_2) to maintain an optimal

114 environment for their performance. However, mixing provided by recirculation pumps in

115 tubular photobioreactors (PBR) and baffles in high rate algae ponds would further increase

116 the energy requirement. A case study carried out in Almería, Spain analysing the cost of

117 operating a 30 m^3 PBR plant found that the use of recirculation pumps and aeration pumps

118 to be, respectively, the first and second highest energy expenders in the operation [18]. A

119 further aspect of a microalgae treatment process is the stage in the treatment train it is

120   introduced.   Traditionally, microalgae remediation has been restricted to polishing

121   secondary treatment effluent – i.e. after the energy intensive secondary treatment stage.

122   Therefore, the introduction of microalgae in such a situation would not result in the much-

123   desired reduction in overall energy demands of wastewater treatment. As described above,

124   this is largely a direct result of additional mixing and aeration provided. In addition, the

125   added cultivation cost is not feasible if the biomass does not compensate for the energy

126   utilised throughout the process. As a result, a more effective treatment process would be to

127   integrate a microalga secondary treatment phase, herein for treating primary settled

128   wastewater (PSW) directly while meeting effluent standards. The application of microalgae

129   would therefore be an alternative biological treatment process to current conventional

130   secondary processes, not just for enhanced removal of N and P.

131   The potential cultivation of microalgae for PSW treatment has, however, not been

132   fully studied in this respect, and a static culturing system could provide a direction for the

133   development of a low energy microalgae treatment system.

134   In this study, we explore the potential for using the microalga *Chlorella vulgaris* to

135   treat municipal PSW and evaluate its efficiency in removing NH_3, PO_4 and chemical oxygen

136   demand (COD) under static culture conditions. To improve the availability of carbon and to

137   overcome potential light limitations caused by the opaque nature of wastewater, the effects

138   of exogenous organic and inorganic carbon on microalgae growth and remediation

139   performance were also evaluated. To validate the efficiency and reproducibility of this

140   process that takes into account natural fluctuations in the composition (biological/chemical)

141   of wastewater, we further conducted three independent batch studies with PSW obtained

142   on different days of the year.

143

2. Materials & Methods

2.1. Microalgae strain, medium and maintenance

Chlorella vulgaris strain CCAP 211/79 was used in all experiments. This is a non-axenic freshwater microalga that was originally isolated from a waste solvent bio-filter at Heriot-Watt University, Edinburgh, UK [19]. All manipulations of the stock culture were carried out under sterile conditions in a biological laminar flow hood to limit the contamination of the culture with other microorganisms.

Strain CCAP 211/79 was maintained in a modified Bold basal medium (BBM, Table S1 and S2) adjusted to pH 7.2 and heat sterilised (121°C, 15 minutes).

Seed cultures used as the inoculum for all experiments were maintained in 350 mL BBM cultured in 500 mL glass bottles which were aerated continuously with atmospheric air through a sterile In-Line HEPA filter (Ø 53 mm, pore size ≥0.3 μm, Whatman International, Ltd, UK) at a volumetric flow rate of 0.15 of air volume per volume of liquid per minute (V/Vm). The cultures were grown in batch mode and sub-cultured at late exponential phase (7 to 9 days). Seed cultures for all experiments were grown for 7 days prior to use as inocula. Environmental growth conditions were the same for both the stock cultures and the experimental runs. These were fixed at 15±1°C and a 12:12 light-dark cycle (Fluora, Osram, Germany) at a photon flux of 100 μmol m^{-2} s^{-1} (US-SQS/L probe, Walz, Germany).

2.2. Wastewater source

Primary settled wastewater was obtained from Seafield Wastewater Treatment Plant located in Edinburgh, UK. The facility treats predominantly domestic wastewater from Edinburgh City and the surrounding area via a combined sewer catchment. The site treats an average flow of 283 ML day^{-1} with a population equivalent of approximately 800,000,

7

168   treated to comply with the carbonaceous treatment standards required by the Urban

169   Wastewater Treatment Directive with a final effluent biological oxygen demand (BOD) and

170   COD less than 25 mg L^{-1} O_2 and 125 mg L^{-1} O_2 respectively [5]. The treatment process

171   comprises of 10 preliminary screens, 4 grit removal tanks, 4 primary settlement clarifiers

172   and a plug flow secondary activated sludge plant with a discharge to the Firth of Forth via a

173   long sea outflow [20].

174   The samples were collected from this same primary settling tank effluent channel for

175   all our experimental work. Wastewater samples were collected fresh on the day an

176   experiment was to be commenced and taken directly to Heriot-Watt University where they

177   were processed within two hours. Prior to use in our experiments, the wastewater was

178   filtered through a Whatman 113 filter (Ø 90mm, pore size 30 μm, Whatman International,

179   Ltd, UK) as a pre-treatment step to provide consistency in turbidity between samples. No

180   sterilization or further treatment was done.

181

182   **2.3. Experimental conditions**

183   **2.3.1. Quantities of organic carbon and inorganic carbon added for enrichment**

184   The amount of organic carbon added to the PSW samples throughout this study was

185   set to generate an equivalent Chemical Oxygen Demand of 300 mg L^{-1} O_2. For glucose this

186   equated to 281.1 mg L^{-1}, whereas for glycerol this was 245.9 mg L^{-1}. Prior to use, D-glucose

187   (as powder) was oven-dried overnight at 105°C. For glycerol, several millilitres were heat

188   sterilised (121°C, 15 minutes), then allowed to cool to room temperature and the quantity

189   required accurately weighed in a pre-weighed Falcon tube. A small amount of wastewater

190   sample was added to the glycerol in the tube in order to reduce its viscosity and facilitate its

191   transfer. In order to recover all of the glycerol in the tube, aliquots of wastewater from the

192 sample were used to wash the tube three times. CO_2 was bubbled directly into the

193 wastewater sample through a sterile In-Line HEPA filter at a rate of 0.2 V/Vm for 1 minute

194 every 8 hours. The gas flow was controlled by a rotameter (FL-2010, Omega Engineering

195 Limited, UK) with injection time regulated by a solenoid valve (CO2Art Ltd, UK) connected to

196 a programmable 24 hour time switch.

197

198 **2.3.2. Initial glucose enrichment experiment**

199   Glucose enrichment in PSW with microalgae was performed in 450 mL of

200 wastewater contained in 500 mL glass bottles. For this, a cell suspension of *C. vulgaris*

201 grown on BBM was concentrated by centrifugation (3500*g*; 10 min) in 50 mL Falcon tubes

202 and washed twice with 10 mL of the collected wastewater. Three litres of filtered PSW was

203 transferred to a 5 litre glass bottle and inoculated with the washed microalgae at a biomass

204 dry weight concentration of 0.1 g L^{-1}. For enrichment, 1.5 litres of the wastewater with *C.*

205 *vulgaris* was transferred to a 2 litre glass bottle and amended with glucose (see section

206 2.3.1.), and then the sample divided between three 500 mL glass bottles. This step was

207 repeated separately for the enrichment of the wastewater only treatment without the

208 addition of the microalga. In total, four conditions, each in triplicate were set up and

209 labelled as follows: Wastewater control (WWC), Wastewater with glucose (WWG),

210 Wastewater with *C. vulgaris* (WW+*C.v*) and Wastewater with glucose and *C. vulgaris*

211 (WWG+*C.v*). The four treatments were incubated for a period of 5 days, and sampling

212 conducted daily to measure microalgal growth, inorganic nutrient concentration and organic

213 analysis of the wastewater. Samples were collected through a tube internalised which were

214 capped prior to sterilisation of glassware.

215

2.3.3. Evaluating the reproducibility of the treatment efficiency by _C. vulgaris_ with carbon enrichment across different PSW samples

To validate the effect of treating PSW enriched with organic carbon utilising _C. vulgaris_, a further three environmental PSW samples (batches) were treated independently. In addition to glucose, the effect of glycerol and CO_2 enrichment was also investigated as additional independent treatments. The volume treated was increased to 950 mL and for each batch of PSW one bottle for each condition was set up. Each treatment was repeated once for each PSW batch overall providing a triplicate run for each treatment. For each PSW batch treated, 4 litres of filtered PSW was transferred to a 5 litre glass bottle and inoculated with washed microalgae (as prepared in section 2.3.2.) at a biomass dry weight concentration of 0.1 g L^{-1}. A 950-mL volume of the wastewater with _C. vulgaris_ was then transferred to each bottle. Glucose and glycerol were added directly to the PSW to the concentrations stated in section 2.3.1. The treatment conditions were labelled as follows: Wastewater control (WWC), Wastewater with _C. vulgaris_ (WW+_C.v_), Wastewater with glucose and _C. vulgaris_ (WWG+_C.v_), Wastewater with glycerol and _C. vulgaris_ (WWGY+_C.v_) and Wastewater with CO_2 and _C. vulgaris_ (WWCO$_2$+_C.v_). The five treatments were incubated for a period of 5 days and the equivalent analysis performed as in section 2.3.2.

2.4. Analytical methods

2.4.1. Microalgae growth

Whatman GF/C filters (Ø 25 mm, pore size = 1.2 μm, Whatman International, Ltd, UK) were used to determine the biomass dry weight. Prior to use, filters were washed and dried overnight (105°C) and then placed in a desiccator to cool before being weighed. For sample analysis, a filter was pre-wetted with Milli-Q water and then a known volume of

240 sample was added, under a constant vacuum. The filter was rinsed with Milli-Q water, dried

241 and allowed to cool before being weighed. The dry weight for biomass was calculated from

242 the difference between the final and initial weights recorded and expressed as mg L^{-1}. Each

243 sample was measured in triplicate on the initial (day 0) and at the termination (day 5) of the

244 experiment.

245 The concentration of *C. vulgaris* cells in liquid was determined by direct counting

246 using a Neubauer improved haemocytometer with a depth of 0.1 mm. Samples were

247 agitated to ensure the microalgae were homogenous prior to taking an aliquot and

248 transferring to a Micro tube (1.5 mL). When necessary, the samples were diluted with Milli-

249 Q water to obtain a cell concentration range that could be counted. To each cell suspension

250 used for counting, Lugols solution (to 0.1% v/v final concentration) was added and allowed

251 to sit for approximately one hour. The treated suspensions were then thoroughly mixed

252 and the cells counted and concentrations expressed as cells mL^{-1}.

253

254 **2.4.2. Analysis of inorganics**

255 Inorganic nutrient analysis was performed in accordance with the methods

256 described in Standard Methods [21]. All chemicals were of analytical grade and prepared in

257 Milli-Q water. Colorimetric changes were recorded using a Genesys 20 spectrophotometer

258 (ThermoScientific, UK).   All wastewater analysis was carried out on samples centrifuged at

259 3500*g* for 10 minutes unless otherwise stated.

260 The working procedures for the following analyses were scaled to a 5 mL sample

261 volume: NH_3-N was quantified by the Phenate method measured at 635 nm (4500-NH_3 F);

262 PO_4-P by the Ascorbic acid method measured at 882 nm (4500-P E); NO_2-N by the

263 Diazotisation method measured at 543 nm (4500-NO_2^- B), and NO_3-N by the Hydrazine

264 reduction method measured at 535 nm (4500-NO$_3^-$ G). Prior to conducting these analyses,

265 each procedure was validated and calibration curves generated. Three check standards

266 were performed daily for each inorganic compound to verify the working procedure and

267 reagents. When needed, samples were diluted to fit within the respective calibration range

268 for each analysis performed. Total nitrogen was quantified for all samples using Hach test

269 kit *LCK238*, following the manufactures guidelines with readings recorded on a DR1900

270 spectrophotometer (Hach, Loveland, CO, USA). All samples, for each analysis, were

271 performed in triplicate.

272

273 **2.4.3. Analysis of organics**

274 For the initial glucose enrichment experiment, the amount of glucose in the sample

275 was quantified using the phenol-sulphuric acid method of DuBois *et al*., (1956). Samples

276 were taken daily and centrifuged (15,000g; 5 min) prior to analysis. Briefly, 0.5 mL samples

277 were each mixed with 0.25 mL of 5% w/v phenol solution in a test tube, then 1.5 mL of

278 >98% sulphuric acid was added. The mixtures were vortexed vigorously and then allowed to

279 stand for 10 minutes prior to spectrophotometric measurement at 490 nm. Each day the

280 analysis was performed, a calibration curve using D-glucose standards between the ranges

281 of 10 to 100 mg L^{-1} was included.

282 Chemical oxygen demand (COD) was measured as a surrogate to the organic carbon

283 concentration analysis. A mercury-free, small scale (2 mL) closed-tube method was used

284 (method D) [23], which determines the COD by ferrous titration with Ferroin indicator after

285 digestion. All samples were filtered through a 0.45 μm cellulose acetate filter prior to

286 digestion in order to analyse for the soluble oxidising fractions only (COD$_S$). A check

287 standard between the concentrations of 100 to 400 mg L^{-1} O$_2$ was included in every

288   analytical run, which were diluted from a 1000 mg L^{-1} O$_2$ stock standard that was prepared

289   by dissolving oven dried (105°C) D-glucose in Milli-Q water (0.93720 g L^{-1}).

290

291   **2.5. Analysis of dissolved oxygen and pH**

292   Dissolved oxygen (DO) was quantified using a LDO101 IntelliCAL™ probe and HQ40D

293   meter following the manufactures guidelines (Hach, Loveland, CO, USA). pH was quantified

294   using a HI1230 pH probe and HI8424 pH meter which was calibrated daily (Hanna

295   Instruments, Inc., UK).

296

297   **2.6. Statistical analysis**

298   Figures were generated using Prism version 6.02 (GraphPad Software, USA) and

299   statistical analysis was performed using SPSS version 22 (IBM Corporation, Armonk, NY).

300   Normality and homogeneity of variances for the data was tested with a Shapiro-Wilk test

301   and Levene's test respectively. Since the data were found not to comply with a normal

302   distribution, the differences in the median of the treatments was statistically analysed by

303   Kruskal-Wallis test followed by pairwise comparison using Dunn's procedure with a

304   Bonferroni correction for multiple comparisons ($p < 0.01$). Unless stated otherwise, the *p*-

305   value reported refers to the comparison of a treatment to the control treatment, WWC.

306   Tests were performed between treatments at the time points stated.

3. Results & Discussion

3.1. Effect of enrichment with glucose

3.1.1. Inorganic nutrient removal

Bioavailable organic carbon, in the form of glucose, had a strong influence on the ability of *C. vulgaris* to remove inorganic nutrients from the PSW. In the case of NH_3-N, this was the most abundant form of nitrogen available to the microalga in the PSW (Figure 1A), and its removal was more effective in wastewater that was enriched with glucose compared to the untreated (no glucose) control. In the WWG+*C.v* treatment, NH_3-N concentration rapidly declined from an initial concentration of 28.9 mg L^{-1} to 4.6 mg L^{-1} at day 1, and reached 0.1 mg L^{-1} at day 2. Conversely, in the WW+*C.v* treatment without enrichment with glucose, concentrations of NH_3-N decreased at a slower rate, reaching 19.6 mg L^{-1} at day 1, after which only a total of 2.1 mg NH_3-N was further removed over the remaining four days. In the treatments without the microalgae, NH_3-N decreased to no more than 19.2 mg L^{-1} in the WWG treatment, and no reduction was recorded in the WWC treatment.

It can therefore be argued that the marked reduction in NH_3-N concentration observed in the WWG+*C.v* treatment is a direct result of the additional organic carbon (as glucose) to the PSW. Inorganic nitrogen assimilation in microalgae is inextricably dependent on organic carbon substrates, requiring carbon skeletons in the form of keto-acids and energy from carbon metabolism in the form of ATP and NADPH [24]. The assimilation and incorporation of ammonium into amino acids is brought about by the evolutionary conserved enzymes glutamine synthetase (GS) and glutamine 2-oxoglutarate amino transferase (GOGAT) [25]. GS fixes ammonium on a glutamate molecule to yield glutamine, and the added amino group then can act as the nitrogen donor to 2-oxoglutarate in the

14

331 reduction-dependent conversion to yield two glutamate compounds catalysed by GOGAT.

332 Further amino acid synthesis uses the carbon compound oxaloacetate in the interconversion

333 of amino nitrogen from glutamate to yield aspartate by aspartate aminotransferase. By this

334 mechanism, the incorporation of ammonium has been shown to increase the demand for

335 tricarboxylic acid cycle (TCA) intermediates in microalgae, with 2-oxogluterate and

336 oxaloacetate being the main metabolites [26]. This demand for carbon, which feeds into

337 the TCA cycle, can be fixed or assimilated through autotrophic or heterotrophic pathways,

338 respectively, of mixotrophic algae like *C. vulgaris*. Therefore, when compared to the other

339 treatments, the significantly higher NH_3-N removal efficiency observed in the WWG+*C.v*

340 treatment can be attributed to higher availability of bioavailable carbon, mainly to *C.*

341 *vulgaris*, herein in the form of glucose (H (3) = 10.421, p = 0.002 at day 1).

342   In wastewater treatment, NH_3-N reduction also occurs through its conversion to

343 NO_2, then into NO_3, and N_2 by nitrification and denitrification respectively. Both the NO_2-N

344 and NO_3-N concentrations were consistently on the border of the detection limit in all the

345 PSW samples from the commencement and duration of these experiments (Figure 1C & 1D).

346 We did not analyse for N_2, so the process of nitrification and denitrification cannot be ruled

347 out from occurring here. However, the likelihood of inorganic nitrogen being removed

348 through its conversion to N_2 will have been limited by various chemical and physical factors

349 associated with the treatments, albeit independently from each other. For all treatments

350 the main limitation will have been the relatively short duration of our experiments (5 days),

351 which was insufficient to allow for a longer generation time needed by nitrifying bacteria in

352 PSW. Additionally the observed pH changes, inorganic carbon and O_2 availability in the

353 treatments (see below) may also have limited these pathways [27]. Furthermore, the

354 removal of NH_3-N to almost below detection limits in the WWG+*C.v* treatment occurred

15

355   within only 2 days, and likely well before nitrification had a chance to begin. The pH

356   increase in the WW+$C.v$ treatment and low inorganic carbon availability will have limited the

357   formation of NO_2-N [28]. Although a small increase in NO_2-N was detected in this treatment

358   (i.e. from 0.02 mg L^{-1} to 0.07 mg L^{-1}), this did not coincide with an equivalent amount of NH_3-

359   N removed over the 5-day duration, indicating that nitrification was not the dominant

360   pathway in reducing the ammonium-nitrogen from the PSW. Inorganic nitrogen

361   concentrations in the control treatments (WWC and WWG) remained fairly constant over

362   the 5-day duration of these experiments, with the exception of NH_3-N showing a slight

363   reduction within the first day in the WWG treatment, but which was not significant (H (3) =

364   10.421, p = 0.307 at day 1). This reduction can be ascribed to a high metabolic activity of

365   the microbial community present in the PSW as a result of the exogenous glucose, which

366   coincided with a decrease in total carbohydrate concentration (Figure 2A). A major

367   limitation to these control treatments was the low concentration of dissolved O_2, which can

368   be attributed to the cultures having been incubated statically (Figure 2C). This will have

369   impacted on the metabolic activity of the endogenous microorganisms in digesting and

370   assimilating inorganic nitrogen compounds or converting them by nitrification and, thus,

371   limiting their removal.

372   PO_4-P was drastically reduced in WWG+$C.v$ from 3.2 mg L^{-1} to 0.1 mg L^{-1} at day 1 and

373   remained at this concentration until the end of the treatment period (Figure 1B) (H (3) =

374   10.385, p = 0.002 at day 1). This was a maximal removal efficiency of 96% within a period of

375   1 day. Notably, this is a far higher recorded rate than reported in previous studies using

376   PSW which had reported removal efficiencies of less than 50% for the same retention time

377   [29–31]. The efficiency of P removal is affected by both abiotic and biotic factors. In pH

378   environments of approximately 9 or above, for example, PO_4^{3-} precipitates as a result of

379 chemically reacting with cations in solution, mostly magnesium and calcium ions [32]. The

380 precise efficiency of this phenomenon is dependent on the phosphorus and cation

381 concentration, as well as temperature [32]. In regards to biotic influences, Beuckels *et al*.

382 (2015) described the assimilation of P into microalgal biomass as dependent on the supply

383 of N. Their study identified that biomass P concentrations were low when the N

384 concentration in the biomass was low because they were grown on N-limited medium,

385 irrespective of the amount of P in the medium. Microalgae have also been reported to

386 assimilate and store phosphorus in a mechanism referred to as 'luxury uptake', which occurs

387 when phosphorus uptake exceeds the metabolic requirements of the microalgae [34].

388 Given the high removal efficiency of NH_3-N under neutral pH in the WWG+*C.v* treatment

389 (Figure 1A & 2D) and exponential growth of *C. vulgaris* (Figure 2B), it can be inferred that

390 the main mechanism for PO_4-P removal was through assimilation by *C. vulgaris* and other

391 microorganisms, such as bacteria, present in the wastewater and/or associated with the

392 microalga mainly for direct metabolic use. In comparison, PO_4-P removal in the WW+*C.v*

393 treatment was a result of its assimilation initially and subsequent precipitation after day 1

394 because of a gradual increase in the pH above 9 (Figure 2D). Here, PO_4-P concentrations

395 decreased from 3.2 mg L^{-1} to 1.7 mg L^{-1} by day 1, and then continued to decrease reaching

396 minimal concentrations of 0.8 mg L^{-1} by day 4. The low removal and consequently

397 assimilation rate of NH_3-N by *C. vulgaris* will have likely influenced the internal N

398 concentration of the microalgae, thus also affecting the assimilation of P in this treatment.

399 However, the continuous removal of phosphorus by the microalgae through luxury uptake

400 after day 1 in the WW+*C.v* treatment cannot be ruled out (Figure 2B). This same trend of a

401 slow decrease in PO_4-P after day 1 was not observed in the WWG treatment despite

402 displaying a similar reduction in NH_3-N and PO_4-P as in the WW+*C.v* treatment. The

403   reduction in PO_4-P concentration in the WWG treatment by day 1 was likely through its

404   assimilation and incorporation by the indigenous microbial community present in the PSW,

405   concurrent with the reduction of NH_3-N. As anoxic conditions developed in the control

406   treatments, aerobic metabolism and degradation of the inorganic compounds will have

407   slowed (Figure 2C). However, as the pH did not increase above 8 in these treatments, no

408   substantial decrease in PO_4-P could be attributed to phosphate precipitation.

409   Comparing the capacity to remove inorganic nitrogen and phosphorus between the

410   treatments, the results indicate that regardless of the treatment condition, with or without

411   enrichment, the microalgae were mainly responsible for the elimination from the PSW. In

412   the control treatments (without microalgae) the most effective decline in NH_3-N and PO_4-P

413   was in the WWG treatment, while WWC exhibited no noteworthy change from the initial

414   concentrations of the PSW. This suggests that the natural microbial community of the PSW

415   alone was not able to effectively remove or convert the inorganic compounds to any great

416   extent under the culture conditions imposed. Although the influence of the microbial

417   community cannot be completely disregarded, with respect to eliminating the inorganic N

418   and P their ability to directly do so is limited. This finding is consistent with previous studies

419   employing microalgae-bacteria co-cultures. For example, Su et al. (2012) investigated the

420   potential of a co-culture composed of wastewater-born algae consortium (majority

421   filamentous blue-green algae) and activated sludge, inoculated at different ratios (w/w) on

422   nutrients removed from pre-treated wastewater. The removal efficiencies of total Kjeldahl

423   nitrogen and PO_4-P removal at day 10 were respectively 95.5% and 93.5% in the 5:1 algae-

424   bacteria co-culture, whereas in the reactor with only sludge the concentrations declined to

425   31.4% and <10% respectively. Ma et al. (2014) directly examined the influence of bacteria

426   removing nutrients from centrate, a waste stream following sludge dewatering, with *C.*

427 *vulgaris* by varying the initial concentration of bacteria in the co-culture. Their results

428 revealed no significant difference in nutrient removal from the wastewater with increasing

429 bacteria concentrations, implying that the presence of bacteria had little effects on the

430 removal of the inorganic compounds, at least within the investigated range. In the present

431 study, the contribution of the bacteria in the microalgae treatments to remove the inorganic

432 N and P may have been limited by the composition of the microbial community and

433 environment of the treatment. Biological nutrient removal from wastewater is dependent

434 on specific microorganisms (i.e. nitrifying, denitrifying and phosphorus accumulating

435 organisms), which are encouraged to grow and function by cycling the wastewater through

436 anaerobic, aerobic and anoxic environments [1,27]. The presence of these microorganisms

437 are naturally low in influent wastewater, inhibited by the high concentration of

438 carbonaceous-BOD in influent and settled wastewater, a situation that would have been

439 exacerbated by the deliberate organic carbon enrichment carried out in the experiments

440 reported here. Without these specific microorganisms the removal of N and P in

441 wastewater treatment tends to be minimal. It can be suggested that the microbial

442 population in the microalgae treatments was not composed of these appropriate or

443 adapted microorganisms to facilitate the N and P removal beyond their metabolic

444 capabilities. Another aspect that may have limited the microbial population in removal of

445 inorganic N and P in the microalgae treatments is the high pH environment, particularly in

446 the WW+*C.v* treatment (Figure 2D). Elevated pH (discussed below) in conjunction with high

447 dissolved oxygen concentration (Figure 2C) in a light environment mediate photo-oxidative

448 destruction of coliform bacteria [37,38].
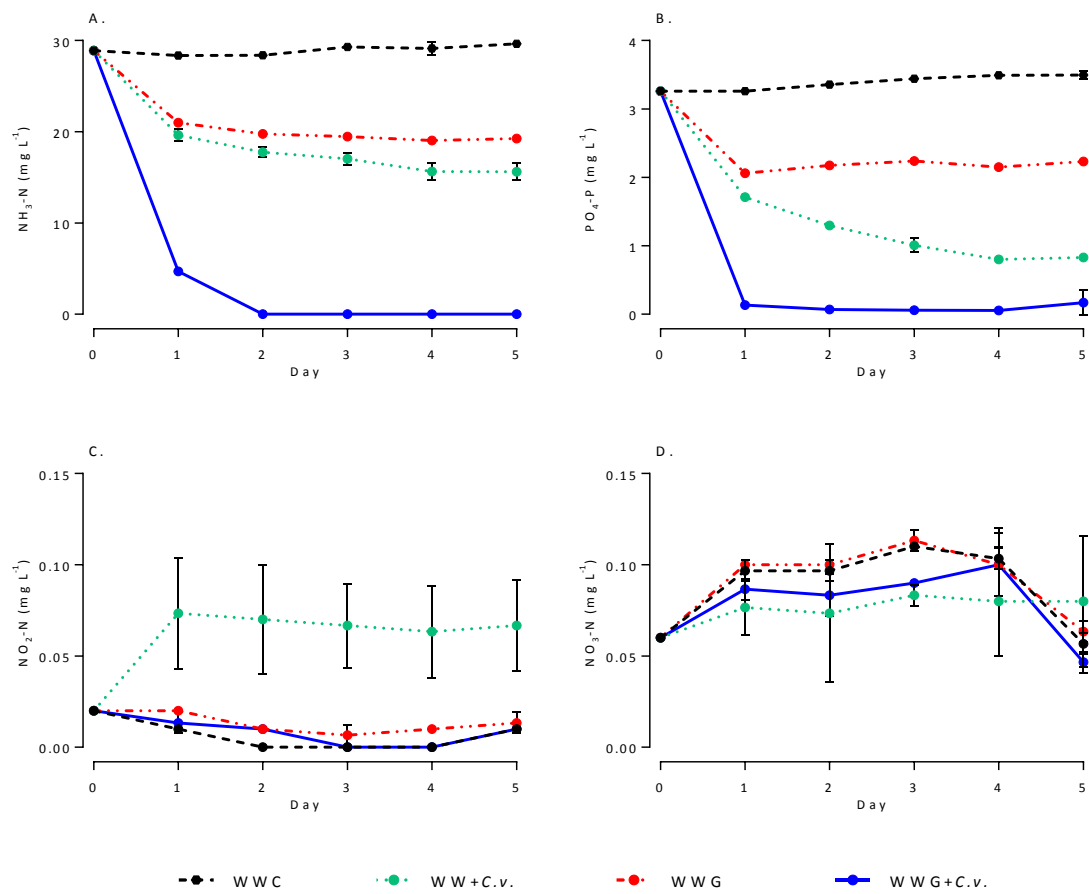
449
450   Figure 1

451

3.1.2. Organic nutrient removal

Under aerobic conditions, organic substrates in wastewater are removed through oxidative biodegradation and incorporation for biosynthesis predominantly by heterotrophic bacteria [1]. Owing to the mixotrophic nature of *C. vulgaris*, it will have participated together with the indigenous bacterial community in the PSW and that associated with the micro-alga, in the collective removal of bioavailable organics from wastewater [39]. Figure 2A shows the total carbohydrate (TC) concentrations for each of the treatments throughout the culture period. Without enrichment with glucose, the initial TC concentration was 9.2 mg L^{-1}, which was lower than the theoretical range of 50 to 120 mg L^{-1} for municipal wastewater, as suggested by Gray (2004). The TC concentration in the WW+*C.v* and WWC treatments declined only slightly to 4.6 mg L^{-1} after 1 day, with no substantial change thereafter. However, in the enriched treatments (WWG and WWG+*C.v*), TC concentration declined rapidly from an initial concentration of 305.1 mg L^{-1} to 9.2 mg L^{-1} after 1 day. It can be inferred that glucose was completely removed within this time since its concentration reached initial concentrations in the non-enriched (WWC) treatment. The COD results further confirm the removal of the glucose from the enriched treatments (Table 1), as shown by a removal of approximately 67% in the WWG and WWG+*C.v* treatments, with final COD readings of 138.3 mg L^{-1} O_2 and 133.6 mg L^{-1} O_2, respectively. These residual COD concentrations suggest that organic compounds in the wastewater could not be metabolised further by the microalgal and bacterial community under the treatment conditions.

Interestingly, the beginning of the *C. vulgaris* stationary growth phase at day 2 in the WWG+*C.v* (Figure 2B) coincided with an increase in TC concentrations (Figure 2A). Henderson *et al.*, (2008) reported an increased production of dissolved organic carbon

476   during the stationary growth phase for various microalgal species, and this was attributed to

477   the excretion of extracellular polysaccharide substances (EPS) by the microalgae. Hence,

478   the observed increase in TC concentrations after day 2 in the WWG+*C.v* treatment could be

479   attributed to EPS production during the stationary phase [41].
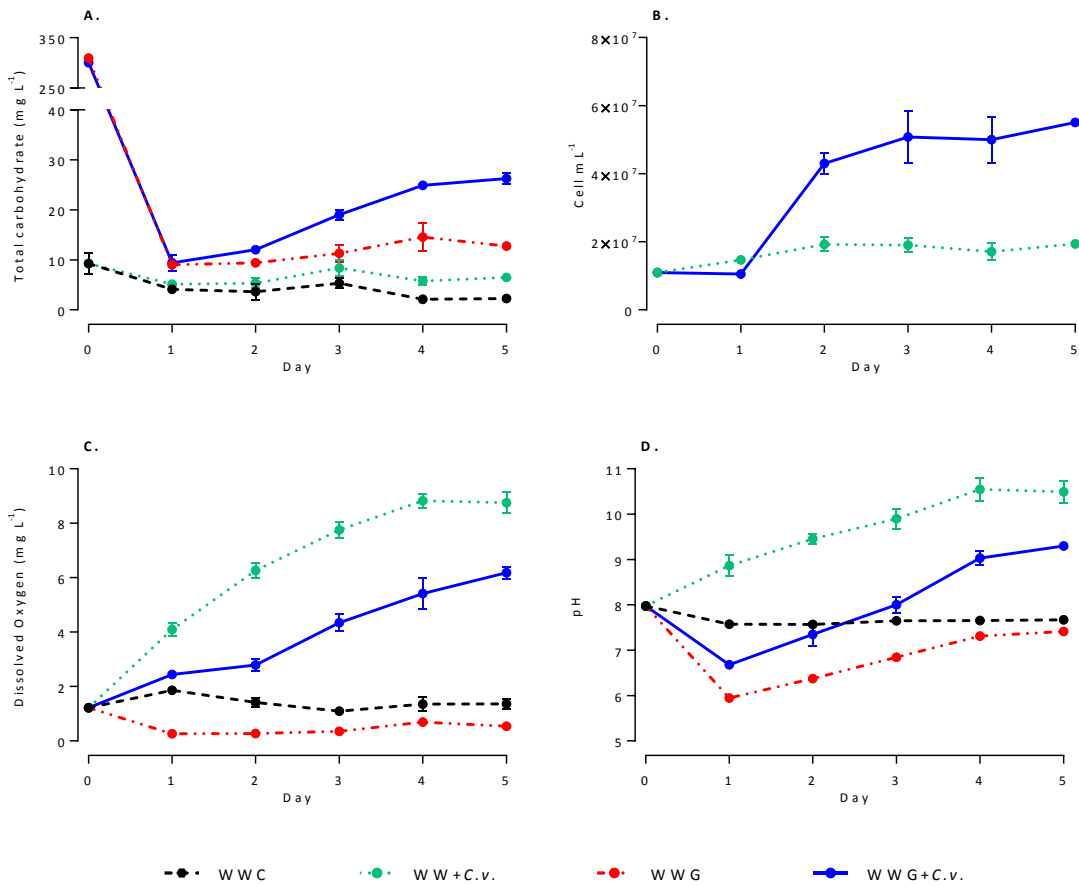
480

481   Figure 2

482

3.1.3. Growth and pH

It was initially hypothesised that indigenous microorganisms, particularly bacteria, in the PSW samples would outcompete *C. vulgaris* for organic and inorganic resources and result in limiting the alga's growth and ability to the remove N, P and the exogenous glucose that was added. Our results, however, indicate that the removal of these components in PSW is enhanced by the inoculation of *C. vulgaris* together with the supplementation of glucose. Indeed, the addition of glucose had a distinctly positive effect on the growth of *C. vulgaris* (treatment WWG+*C.v*) compared to no substantial growth observed in the absence of glucose (treatment WW+*C.v*) (Figure 2B). Although cell count in the WW+*C.v* treatment did not indicate any growth of the microalgae by cell numbers, the biomass measurements were seven times higher compared to that in the WWC treatment which did not contain glucose and was not inoculated with the alga, with dry weights of 280.8 mg L^{-1} and 42.8 mg L^{-1} for the treatments respectively. The WWG+*C.v* treatment had the highest biomass yield with 419.1 mg L^{-1} compared to 111.7 mg L^{-1} for the WWG treatment.

Variations in pH occurred in all four treatments, with the highest degree of change observed in the WW+*C.v* treatment (Figure 2D). The alkalisation of the PSW in this treatment, and in any microalgal culture can be described as a consequence of the fixation of CO_2 by RuBisCO, which is converted from HCO_3^-. This photosynthetic-driven process leaves OH^- ions in the cell which have to be neutralised with H^+ ions that are taken up from the extracellular environment, resulting in an increased extracellular pH [42]. The knock-on effect is a decrease in the CO_2 to bicarbonate ratio, and eventually a reduced absolute CO_2 concentration. As we employed a static culture system, the contribution of atmospheric CO_2 will have been negligible.

506   Furthermore, the unfavourable (high pH) environment present may also have limited

507   the growth of other members of the microbial community in the PSW and thus reduced

508   their production of CO_2 via respiration that would have otherwise served *C. vulgaris* with an

509   alternative source of this essential compound for photosynthesis. Additionally, the pH rise

510   in the WW+*C.v* treatment will have had a strong influence on its NH_3-N removal efficiency

511   (Figure 1A).   While ammonium (NH_4^+) is the preferred inorganic nitrogen source for

512   microalgae, a rise in pH above 8 leads to its dissociation to form free ammonia (NH_3) which

513   is toxic to microalgae and other aquatic organisms [43]. The pH in this treatment increased

514   from 7.97 to 10.49 at a relatively constant rate over the 5-day duration of these

515   experiments (Figure 2D). This will have contributed to the formation of free ammonia

516   creating an unfavourable environment for nutrient assimilation and microalgae growth. The

517   alkalisation also suggests a reduction and consequent limitation in inorganic carbon because

518   of its ability to buffer pH changes in the medium environment. The resultant drop in NH_3-N

519   removal after day 1 in the treatment supports the lack of available carbon before the onset

520   of ammonia toxicity, most likely because of the low inorganic carbon to the microalgae will

521   have limited the assimilation of NH_3-N, as described above (section 3.1.1).

522   Conversely, the pH in the glucose-enriched treatments decreased rapidly within the

523   first day to below 6.6 for WWG+*C.v* and 5.9 for WWG (Figure 2D). This drop in pH coincided

524   with the removal of the added glucose in both treatments (Figure 2A), suggesting that

525   acidification of the PSW did not negatively affect the consumption of this substrate. The

526   anoxic environment in the WWG treatment (Figure 2C) will have driven the degradation of

527   organic compounds, including glucose, to produce organic acids through the process of

528   acidogenesis and acetogenesis and thus the observed pH reduction in this treatment [27].

529   It should also be noted that the pronounced removal of NH_3-N and PO_4-P will have also

24

530   influenced the overall extracellular H^+ concentration and thus influencing the observed

531   shifts in the pH.

532

533   **3.2. Treatment reproducibility assessed across environmental samples and alternative**

534   **carbon sources**

535   The small-scale treatment of PSW with exogenously added glucose was used to evaluate the

536   growth of *C. vulgaris*, its removal of inorganic compounds, and to analyse for other

537   biochemical and physical changes under the different treatment regimens evaluated. This

538   provided a useful understanding of the treatment performance under static culturing

539   conditions revealing that it was limited, either because of the limited bioavailability of

540   carbon to the microalga or detrimental effects from pH changes. In order to upscale this

541   into a commercially-viable system, we would need to demonstrate that this process can be

542   consistently replicated with PSW collected at any time to take into consideration biotic and

543   abiotic variability of the wastewater throughout the year. To investigate this, a further

544   three batches of PSW were collected and treated separately and sequentially with *C.*

545   *vulgaris* employing the same static culturing approach as described and evaluated above. In

546   addition to enriching with glucose, treatments with glycerol and CO_2 were also included to

547   compare between the use of a different organic and inorganic carbon source.

548   Figure 3 shows the average percentage removal efficiency for NH_3-N and PO_4-P for

549   each treatment from the three batches of PSW combined. Overall, the efficiency in NH_3-N

550   and PO_4-P removal across the batches of PSW was effective and reliable in the treatments

551   with exogenous organic carbon. The treatments enriched with glucose and glycerol

552   performed the same with respect to their removal of NH_3-N and PO_4-P, with a respective

553   91% and 98% average efficiency in both treatments (both $p < 0.01$ at day 2). In comparison,

554 WWCO$_2$+*C.v* had an average removal efficiency of 55% for NH$_3$-N and 64% for PO$_4$-P. The

555 acidification of the medium in the WWCO$_2$+*C.v* treatment is the most likely reason that

556 caused the decreased removal efficiency in NH$_3$-N and PO$_4$-P compared to the organic

557 carbon enriched treatments (Figure 4B, R1 – R3)). Despite the limited sparging of CO$_2$, the

558 aqueous dissolved CO$_2$ in this treatment resulted in a pH drop to approximately 5.5 after

559 day 2, which may have adversely affected growth of the microalga. The presence of excess

560 CO$_2$ available to the microalga was to enhance photosynthetic productivity.   However,

561 microalgal growth itself was limited in this treatment showing a similar growth pattern and

562 cell concentration as in the WW+*C.v* treatment which had no form of enrichment (Figure 4A

563 R1 – R3)).   It has been suggested that excess CO$_2$ concentrations can lower or inhibit

564 microalgae respiration because of its strong influence on photosynthetic efficiency [44].

565 This may, hence, explain the observed lower growth in this treatment condition. Similarly,

566 NO$_2$-N and NO$_3$-N concentrations between the treatment types showed no substantial or

567 detectable change (Figure S1).   However, small differences in the initial concentration

568 between the PSW batches of these inorganic nitrogen compounds was recorded, although

569 this seemed to have little effect on the overall performance of the process.
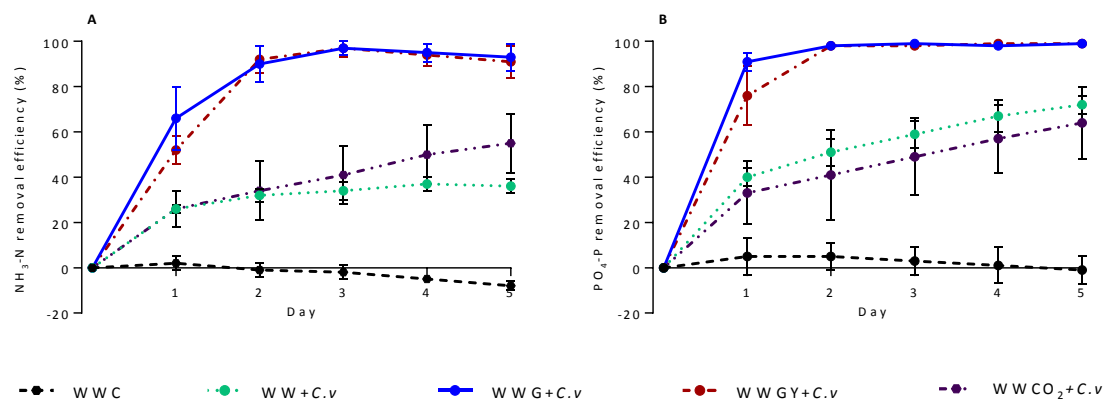
570
571

572   Figure 3

573

Figure 4

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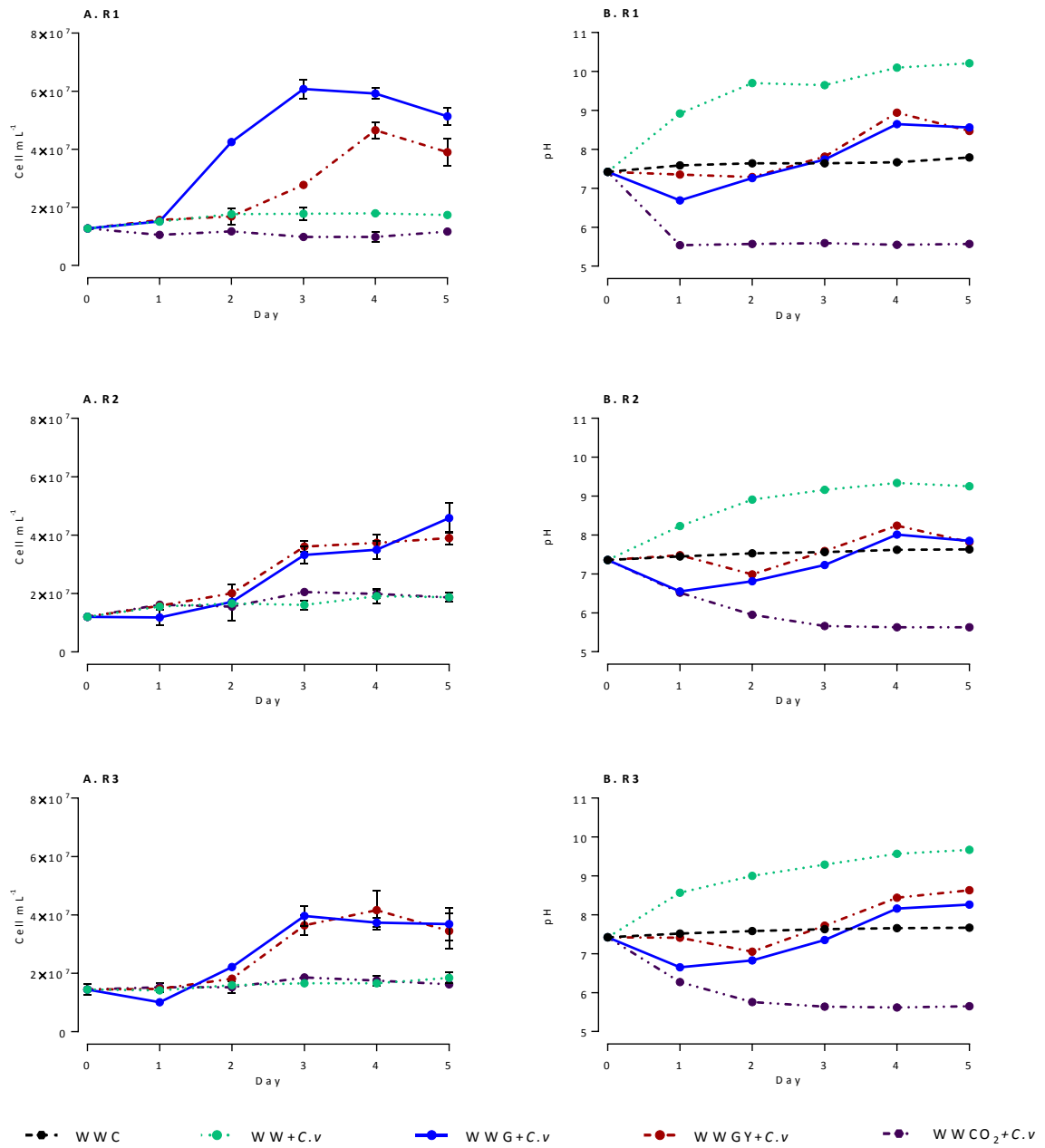
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578   The final effluent concentrations from a wastewater treatment system are a key

579   criteria in validating the performance of the process. Meeting final discharge maximums set

580   at the more restrictive limit of 10 mg L^{-1} TN, 1 mg L^{-1} TP and 125 mg L^{-1} COD laid out by the

581   Urban Wastewater Treatment Directive are preferable [5]. Although the organic carbon-

582   enriched treatments removed an average of >90% of NH_3-N and PO_4-P, between the three

583   batches of PSW that were treated, the variation in the initial concentration of these

584   inorganic compounds in each PSW batch effect the efficiency of their removal. Batch 2 and

585   3 had the highest concentration of TN compared to batch 1 (Table 2). This impacted on the

586   final TN effluent concentration, as a higher initial concentration led to a higher final

587   concentration (Figure 5 & Table S3). For batch 3, final TN was >11 mg L^{-1} in both the glucose

588   and glycerol enriched treatments, and COD >125 mg L^{-1} O_2 in the glucose enriched

589   treatment. This suggests that there is a limitation between the maximum N concentrations

590   that could be treated in the presence of the enriched carbon quantity added to the PSW

591   batches in this study.   To explore this further, an experiment with PSW and the

592   concentration of organic carbon used throughout this study with controlled N ratios would

593   need to be carried out under static culturing to further validate this effect. The maximum

594   microalgal cell concentrations reached were also affected, which were lower in batches 2

595   and 3 (Figure 4A, R2 & R3). *C. vulgaris* increased to > 4.5 x 10^7 cells mL^{-1} in batch 1, with a

596   maximum cell concentration of 6.08 x 10^7 and 4.65 x 10^7 cells mL^{-1} for the treatments

597   enriched with glucose and glycerol, respectively. In batches 2 and 3, the maximum cell

598   concentration reached in either of these organic carbon enrichment treatments was < 4.5 x

599   10^7 cells mL^{-1}.

600   Future work could evaluate an alternative source of organic carbon to determine its

601   impact on PSW treatment with *C. vulgaris* under the static co-culture treatment process

602 used from laboratory setting to commercial application. Despite the low quantities of

603 glucose or glycerol used this is not cost effective at a commercial scale therefore, an

604 alternative organic carbon sources ideally from a waste sources is needed to substitute for

605 their use [45]. Optimisation of the process to mitigate the fluctuations in pH could also be

606 explored and potentially easily overcome with the use of an appropriate photo-bioreactor

607 design, preferably incorporating a semi-continuous treatment process.
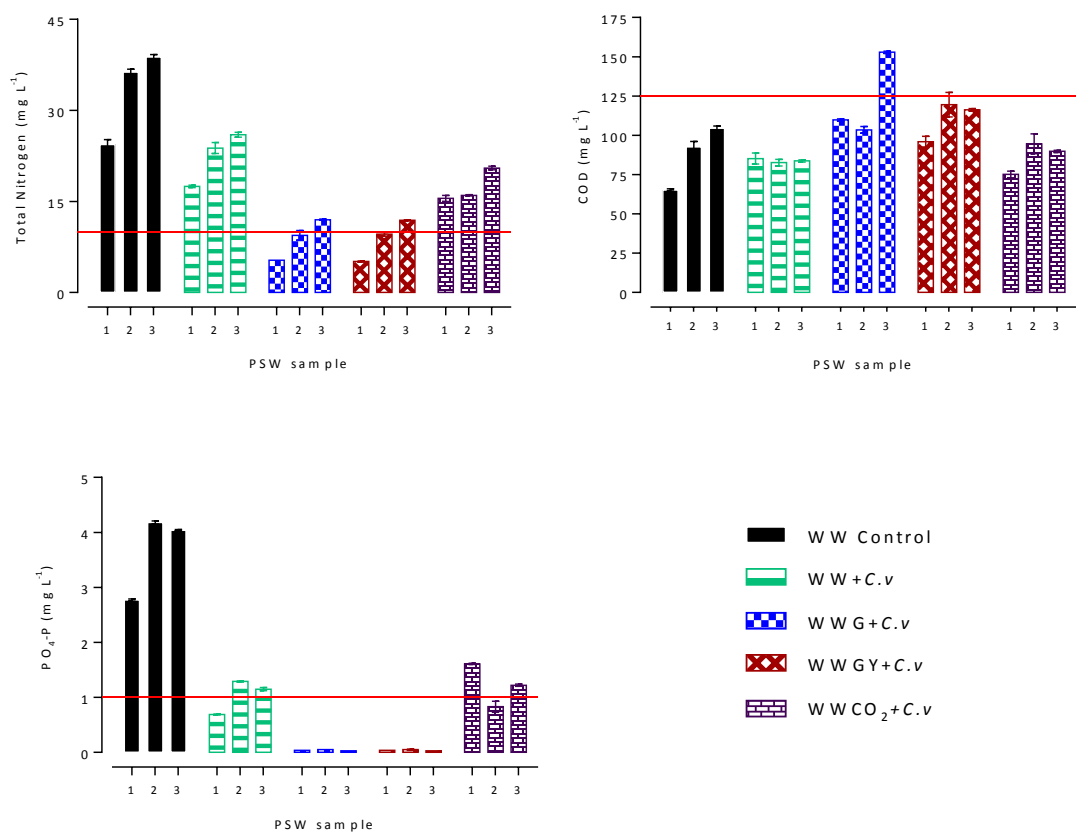
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609 Figure 5

610

4. Conclusion

This study aimed to evaluate the influence of organic carbon enrichment on *C. vulgaris* performance in order to reduce both the carbonaceous and inorganic nutrient load in PSW under static cultivation conditions. Initial experiments with glucose enrichment demonstrated a significant removal of NH_3-N and PO_4-P in the WWG+*C.v* treatment, from a concentration of 28.9 to 0.1 mg L^{-1} and 3.2 to 0.1 mg L^{-1} respectively. The rate of removal compared to the WW+*C.v* treatment was attributed to the higher availability of carbon that we suspect supported the microalga's TCA cycle. No significant formation of NO_3-N and NO_2-N was detected, indicating that nitrification activity was limited in these treatments for various reasons, albeit independently from each other. Performance of the treatment process was replicated on a further three batches of PSW, either enriched with glucose, glycerol or CO_2. For all PSW batches, organic carbon enrichment with *C. vulgaris* resulted in a consistent rate of reduction (>90%) of NH_3-N and PO_4-P, irrespective of the initial concentration of these inorganics in the wastewater. However, higher initial concentrations of these inorganics did not lead to their reduction to levels as low as those achieved when their initial concentrations were lower, hence suggesting that the capacity of the microalgae in this respect for treating PSW may be limited by the availability of organic carbon. Overall, NH_3-N, PO_4-P and COD reduction in the carbon-enriched PSW treatments with the *C. vulgaris* was achieved in a relatively short time (2 days) and at a lower temperature in comparison to previous studies. The application of *C. vulgaris* to treat PSW without aeration offers a key area to develop low energy biological wastewater treatment compared to conventional secondary processes.

30

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Figure Captions

779 **Figure 1** Changes in the PSW concentrations for NH_3-N (A), PO_4-P (B), NO_2-N (C) and NO_3-N

780 (D) in mg L^{-1} treated with and without *C. vulgaris*, enriched with or without glucose. Each

781 data point is the mean ± SD, n = 3. Some error bars are smaller than the symbol. Treatment

782 WWC (wastewater only); Treatment WW+*C.v* (wastewater with *C. vulgaris*); Treatment

783 WWG (wastewater with glucose); Treatment WWG+*C.v* (wastewater with glucose and *C.*

784 *vulgaris*).

785

786 **Figure 2** Comparison of growth in *Chlorella* (B) used to bioremediate PSW enriched or not

787 with glucose, and changes in total carbohydrate (A), dissolved oxygen in mg L^{-1} (C) and pH

788 (D) for each treatment for the duration of the experiment. Data points are mean ± SD, n =

789 3.

790

791 **Figure 3** Percentage removal efficiency of NH_3-N (A) and PO_4-P (B) averaged from the three

792 batches of PSW treated with and without *C. vulgaris*, enriched or not with either glucose,

793 glycerol or CO_2. Data points are mean ± SD, n = 3 (1 for each batch of PSW). Treatment

794 WWC (wastewater only); Treatment WW+*C.v* (wastewater with *C. vulgaris*); Treatment

795 WWG+*C.v* (wastewater with glucose and *C. vulgaris*); Treatment WWGY+*C.v* (wastewater

796 with glycerol and *C. vulgaris*); Treatment WWCO$_2$+*C.v* (wastewater with CO_2 and *C. vulgaris*).

797

798 **Figure 4** Cell concentration (cell mL^{-1}) (A) and pH (B) for each PSW batches treated under the

799 conditions with and without *C. vulgaris*, enriched or not with either glucose, glycerol or CO_2.

800 Cell concentration is an average of three counts (pseudo replicate for each batch of PSW)

801 and pH from one measurement from each treatment. R1, R2 and R3 correspond to PSW

802 batch sample 1, 2 and 3 respectively.

803

804 **Figure 5** Final effluent characteristics for each of the separate PSW batches are presented

805 with 1, 2 and 3 corresponding to the separate batch samples. Red lines indicate the stricter

806 discharge limits permissible by EU law [5]. The 1 mg L^{-1} limit for PO_4-P does not represent

807 the true limit as this is set for TP which was not analysed.

808

Condition	Initial COD_S	Final COD_S
WW	141.9 ± 4.2	101.6 ± 5.6
WWG	416.3 ± 15	138.3 ± 3.1
WW+*C.v*	141.9 ± 4.2	106.6 ± 8.4
WWG+*C.v*	422.4 ± 5.8	133.6 ± 9.1

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814 **Table 1** Chemical Oxygen Demand (soluble) concentrations for PSW in the four treatments

815 in the initial glucose enriched experiment. Values are mean ± SD, n = 3 reported as mg L^{-1}

816 O_2 for the initial composition of PSW with exogenous glucose and for the final readings

817 taken on day 5.

	NH_3-N	PO_4-P	NO_2-N	NO_3-N	COD_S	pH	TN
Batch 1	23.4 ± 0.2	2.9 ± 0.1	0.30 ± 0.0	0.41 ± 0.0	113.9 ± 5.3	7.42	29.8 ± 0.2
Batch 2	34.9 ± 0.5	4.3 ± 0.3	0.03 ± 0.0	0.06 ± 0.0	219.6 ± 10.0	7.36	38.7 ± 1.8
Batch 3	34.7 ± 0.2	3.7 ± 0.1	0.03 ± 0.0	0.06 ± 0.0	182.0 ± 6.1	7.42	44.5 ± 0.7

818

819 **Table 2** Physiochemical characteristics of the three batches of PSW used in the experiment

820 to validate the reproducibility of the static treatment process, analysis from centrifuged

821 samples. Concentrations recorded in mg L^{-1}.