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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 ( $< 300 \text{ mg L}^{-1}$ ) as an organic carbon source to facilitate the bioremediation by the mixotrophic microalga *Chlorella vulgaris*. Characterisation of the wastewater revealed significant reductions in  $\text{NH}_3\text{-N}$  (from  $28.9$  to  $0.1 \text{ mg L}^{-1}$ ) and  $\text{PO}_4\text{-P}$  (from  $3.2$  to  $0.1 \text{ mg L}^{-1}$ ) 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  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$  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 ( $\text{CO}_2$ ) carbon sources. The efficiency of the microalgae treatment process at reducing  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$  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.

### 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 ( $\text{O}_2$ ) to enable the endogenous microorganisms present to breakdown and assimilate the organic and inorganic matter. This stipulation for  $\text{O}_2$  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  $1 \text{ mg L}^{-1}$  total phosphorus (TP) for population equivalence of  $< 100 \text{ k}$  or  $> 100 \text{ k}$ , 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  $< 0.5 \text{ mg L}^{-1}$  is necessary and currently under consideration [8].

In recent decades, policies to safeguard water resources have

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influenced the development of wastewater treatment systems and its management, including a focus on energy consumption and the sustainable performance of these industrial processes. Given the importance of wastewater treatment, a key question is how to reduce energy consumption of this process without affecting performance in respect to meeting water discharge limits. One direction towards making wastewater treatment more sustainable is to recover the resources that it holds, such as water, nutrients (e.g. P and N) and energy. Verstraete et al., (2009) estimated the total value of resources which could be recovered from wastewater at € 0.35 per m<sup>3</sup> based on 2009 market prices [9]. The shift of wastewater treatment from being an end-of-pipeline process to a resource has seen the development and operation of technologies such as sludge digestion for methane production, the integration of energy capturing technology utilising the wastewater treatment infrastructure and nutrient recovery aimed at P and N [2,10].

One particular option for the remediation and capture of inorganic N and P from wastewater is using microalgae. The rationale behind this approach lies in the ability of mixotrophic microalgae to utilise organic and inorganic carbon, as well as the N and P in wastewater for their growth, hence leading to a reduction in the concentration of these substances that will meet discharge limits. Simultaneously, energy-rich microalgal biomass is produced that could be recovered and utilised for the generation of energy or other products following further processing. The remediation potential of this approach has been evaluated for use in an array of wastewater types with promising results [11,12]. A further benefit of microalgae incorporation into wastewater treatment is their generation of dissolved O<sub>2</sub> via photosynthesis. Photosynthetic oxygenation has the potential to meet dissolved O<sub>2</sub> needs to a treatment system without the use of mechanical aeration or mixing, thereby reducing the energy demand for the treatment process. To exemplify, Karya et al. (2013) employed a sequence batch design with *Scenedesmus* sp. and nitrifying bacteria isolated from activated sludge to evaluate whether this co-culture system can support nitrification [13]. Without mechanical aeration, the process was shown successful in reducing 81 to 85% of ammonium-nitrogen through its conversion to nitrate-nitrogen by nitrification, for which the O<sub>2</sub> for this process had been generated by the microalga. Further support for a microalgae-based wastewater treatment approach as a viable biological system relates to its general improved performance in the presence of bacteria. Although considered unavoidable and a major challenge because of the potential to out-compete algae, the presence of bacteria in co-culture with mixotrophic microalgae has been shown to respond better in treating wastewater compared to the use of axenic cultures [14,15]. This affect has been attributed to the exchange of co-factors between the microalgae and bacteria, which include growth promoting compounds and vitamins [16]. Furthermore, when compared to current secondary treatment systems, microalgae also provide a potential system for sequestering carbon as well as removal of micro-pollutants and toxic metals [17].

Despite these advantages, there are various practical and economic challenges that still limit the implementation of microalgae-bacteria co-cultures for wastewater treatment. One such challenge is the cultivation process. As with most conventional wastewater treatment operations, aeration systems are used in microalgae culturing to provide mixing for improving the exchange of O<sub>2</sub> and carbon dioxide (CO<sub>2</sub>) to maintain an optimal environment for their performance. However, mixing provided by recirculation pumps in tubular photobioreactors (PBR) and baffles in high rate algae ponds would further increase the energy requirement. A case study carried out in Almería, Spain analysing the cost of operating a 30 m<sup>3</sup> PBR plant found that the use of recirculation pumps and aeration pumps to be, respectively, the first and second highest energy exponders in the operation [18]. A further aspect of a microalgae treatment process is the stage in the treatment train it is introduced. Traditionally, microalgae remediation has been restricted to polishing secondary treatment effluent – i.e. after

the energy intensive secondary treatment stage. Therefore, the introduction of microalgae in such a situation would not result in the much-desired reduction in overall energy demands of wastewater treatment. As described above, this is largely a direct result of additional mixing and aeration provided. In addition, the added cultivation cost is not feasible if the biomass does not compensate for the energy utilised throughout the process. As a result, a more effective treatment process would be to integrate a microalga secondary treatment phase, herein for treating primary settled wastewater (PSW) directly while meeting effluent standards. The application of microalgae would therefore be an alternative biological treatment process to current conventional secondary processes, not just for enhanced removal of N and P. The potential cultivation of microalgae for PSW treatment has, however, not been fully studied in this respect, and a static culturing system could provide a direction for the development of a low energy microalgae treatment system.

In this study, we explore the potential for using the microalga *Chlorella vulgaris* to treat municipal PSW and evaluate its efficiency in removing NH<sub>3</sub>, PO<sub>4</sub> and chemical oxygen demand (COD) under static culture conditions. To improve the availability of carbon and to overcome potential light limitations caused by the opaque nature of wastewater, the effects of exogenous organic and inorganic carbon on microalgae growth and remediation performance were also evaluated. To validate the efficiency and reproducibility of this process that takes into account natural fluctuations in the composition (biological/chemical) of wastewater, we further conducted three independent batch studies with PSW obtained on different days of the year.

## 2. Materials and 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 min).

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<sup>-2</sup> s<sup>-1</sup> (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 municipal wastewater from Edinburgh City and the surrounding area via a combined sewer catchment. The site treats an average flow of 283 ML day<sup>-1</sup> with a population equivalent of approximately 800,000, treated to comply with the carbonaceous treatment standards required by the Urban Wastewater Treatment Directive with a final effluent biological oxygen demand (BOD) and COD of < 25 mg L<sup>-1</sup> O<sub>2</sub> and 125 mg L<sup>-1</sup> O<sub>2</sub> respectively [5]. The treatment process comprises of 10 preliminary screens, 4 grit removal tanks, 4 primary settlement

clarifiers and a plug flow secondary activated sludge plant with a discharge to the Firth of Forth via a long sea outflow [20].

The samples were collected from this same primary settling tank effluent channel for all our experimental work. Wastewater samples were collected fresh on the day an experiment was to be commenced and taken directly to Heriot-Watt University where they were processed within two hours. Prior to use in our experiments, the wastewater was filtered through a Whatman 113 filter ( $\varnothing$  90 mm, pore size 30  $\mu$ m, Whatman International, Ltd., UK) as a pre-treatment step to provide consistency in turbidity between samples. No sterilization or further treatment was done.

### 2.3. Experimental conditions

#### 2.3.1. Quantities of organic carbon and inorganic carbon added for enrichment

The amount of organic carbon added to the PSW samples throughout this study was set to generate an equivalent Chemical Oxygen Demand of 300 mg L<sup>-1</sup> O<sub>2</sub>. For glucose this equated to 281.1 mg L<sup>-1</sup>, whereas for glycerol this was 245.9 mg L<sup>-1</sup>. Prior to use, D-glucose (as powder) was oven-dried overnight at 105 °C. For glycerol, several millilitres were heat sterilised (121 °C, 15 min), then allowed to cool to room temperature and the quantity required accurately weighed in a pre-weighed Falcon tube. A small amount of wastewater sample was added to the glycerol in the tube in order to reduce its viscosity and facilitate its transfer. In order to recover all of the glycerol in the tube, aliquots of wastewater from the sample were used to wash the tube three times. CO<sub>2</sub> was bubbled directly into the wastewater sample through a sterile In-Line HEPA filter at a rate of 0.2 V/Vm for 1 min every 8 h. The gas flow was controlled by a rotameter (FL-2010, Omega Engineering Limited, UK) with injection time regulated by a solenoid valve (CO2Art Ltd., UK) connected to a programmable 24 h time switch.

#### 2.3.2. Initial glucose enrichment experiment

Glucose enrichment in PSW with microalgae was performed in 450 mL of wastewater contained in 500 mL glass bottles. For this, a cell suspension of *C. vulgaris* grown on BBM was concentrated by centrifugation (3500g; 10 min) in 50 mL Falcon tubes and washed twice with 10 mL of the collected wastewater. Three litres of filtered PSW was transferred to a 5 L glass bottle and inoculated with the washed microalgae at a biomass dry weight concentration of 0.1 g L<sup>-1</sup>. For enrichment, 1.5 L of the wastewater with *C. vulgaris* was transferred to a 2 L glass bottle and amended with glucose (see Section 2.3.1.), and then the sample divided between three 500 mL glass bottles. This step was repeated separately for the enrichment of the wastewater only treatment without the addition of the microalga. In total, four conditions, each in triplicate were set up and labelled as follows: Wastewater control (WWC), Wastewater with glucose (WWG), Wastewater with *C. vulgaris* (WW + *C.v*) and Wastewater with glucose and *C. vulgaris* (WWG + *C.v*). The four treatments were incubated for a period of 5 days, and sampling conducted daily to measure microalgal growth, inorganic nutrient concentration and organic analysis of the wastewater. Samples were collected through a tube internalised which were capped prior to sterilization of glassware.

#### 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<sub>2</sub> 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 L of

filtered PSW was transferred to a 5 L 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<sup>-1</sup>. 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<sub>2</sub> and *C. vulgaris* (WWCO<sub>2</sub> + *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 ( $\varnothing$  25 mm, pore size = 1.2  $\mu$ m, 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 sample was added, under a constant vacuum. The filter was rinsed with Milli-Q water, dried and allowed to cool before being weighed. The dry weight for biomass was calculated from the difference between the final and initial weights recorded and expressed as mg L<sup>-1</sup>. Each sample was measured in triplicate on the initial (day 0) and at the termination (day 5) of the experiment.

The concentration of *C. vulgaris* cells in liquid was determined by direct counting using a Neubauer improved haemocytometer with a depth of 0.1 mm. Samples were agitated to ensure the microalgae were homogenous prior to taking an aliquot and transferring to a Micro tube (1.5 mL). When necessary, the samples were diluted with Milli-Q water to obtain a cell concentration range that could be counted. To each cell suspension used for counting, Lugols solution (to 0.1% v/v final concentration) was added and allowed to sit for approximately one hour. The treated suspensions were then thoroughly mixed and the cells counted and concentrations expressed as cells mL<sup>-1</sup>.

#### 2.4.2. Analysis of inorganics

Inorganic nutrient analysis was performed in accordance with the methods described in Standard Methods [21]. All chemicals were of analytical grade and prepared in Milli-Q water. Colorimetric changes were recorded using a Genesys 20 spectrophotometer (ThermoScientific, UK). All wastewater analysis was carried out on samples centrifuged at 3500g for 10 min unless otherwise stated.

The working procedures for the following analyses were scaled to a 5 mL sample volume: NH<sub>3</sub>-N was quantified by the Phenate method measured at 635 nm (4500-NH<sub>3</sub> F); PO<sub>4</sub>-P by the Ascorbic acid method measured at 882 nm (4500-P E); NO<sub>2</sub>-N by the Diazotisation method measured at 543 nm (4500-NO<sub>2</sub><sup>-</sup> B), and NO<sub>3</sub>-N by the Hydrazine reduction method measured at 535 nm (4500-NO<sub>3</sub><sup>-</sup> G). Prior to conducting these analyses, each procedure was validated and calibration curves generated. Three check standards were performed daily for each inorganic compound to verify the working procedure and reagents. When needed, samples were diluted to fit within the respective calibration range for each analysis performed. Total nitrogen was quantified for all samples using Hach test kit LCK238, following the manufactures guidelines with readings recorded on a DR1900 spectrophotometer (Hach, Loveland, CO, USA). All samples, for each analysis, were performed in triplicate.

#### 2.4.3. Analysis of organics

For the initial glucose enrichment experiment, the amount of glucose in the sample was quantified using the phenol-sulphuric acid method of DuBois et al. [22]. Samples were taken daily and centrifuged (15,000g; 5 min) prior to analysis. Briefly, 0.5 mL samples were each

mixed with 0.25 mL of 5% wv phenol solution in a test tube, then 1.5 mL of > 98% sulphuric acid was added. The mixtures were vortexed vigorously and then allowed to stand for 10 min prior to spectrophotometric measurement at 490 nm. Each day the analysis was performed, a calibration curve using D-glucose standards between the ranges of 10 to 100 mg L<sup>-1</sup> was included.

Chemical oxygen demand (COD) was measured as a surrogate to the organic carbon concentration analysis. A mercury-free, small scale (2 mL) closed-tube method was used (method D) [23], which determines the COD by ferrous titration with Ferroin indicator after digestion. All samples were filtered through a 0.45 µm cellulose acetate filter prior to digestion in order to analyse for the soluble oxidising fractions only (COD<sub>s</sub>). A check standard between the concentrations of 100 to 400 mg L<sup>-1</sup> O<sub>2</sub> was included in every analytical run, which were diluted from a 1000 mg L<sup>-1</sup> O<sub>2</sub> stock standard that was prepared by dissolving oven dried (105 °C) D-glucose in Milli-Q water (0.93720 g L<sup>-1</sup>).

### 2.5. Analysis of dissolved oxygen and pH

Dissolved oxygen (DO) was quantified using a LDO101 IntelliCAL™ probe and HQ40D meter following the manufactures guidelines (Hach, Loveland, CO, USA). pH was quantified using a HI1230 pH probe and HI8424 pH meter which was calibrated daily (Hanna Instruments, Inc., UK).

### 2.6. Statistical analysis

Figures were generated using Prism version 6.02 (GraphPad Software, USA) and statistical analysis was performed using SPSS version 22 (IBM Corporation, Armonk, NY). Normality and homogeneity of variances for the data was tested with a Shapiro-Wilk test and Levene's test respectively. Since the data were found not to comply with a normal distribution, the differences in the median of the treatments was statistically analysed by Kruskal-Wallis test followed by pairwise comparison using Dunn's procedure with a Bonferroni correction for multiple comparisons ( $p < 0.01$ ). Unless stated otherwise, the  $p$ -value reported refers to the comparison of a treatment to the control treatment, WWC. Tests were performed between treatments at the time points stated.

## 3. Results and discussion

### 3.1. Effect of enrichment with glucose

#### 3.1.1. Inorganic nutrient removal

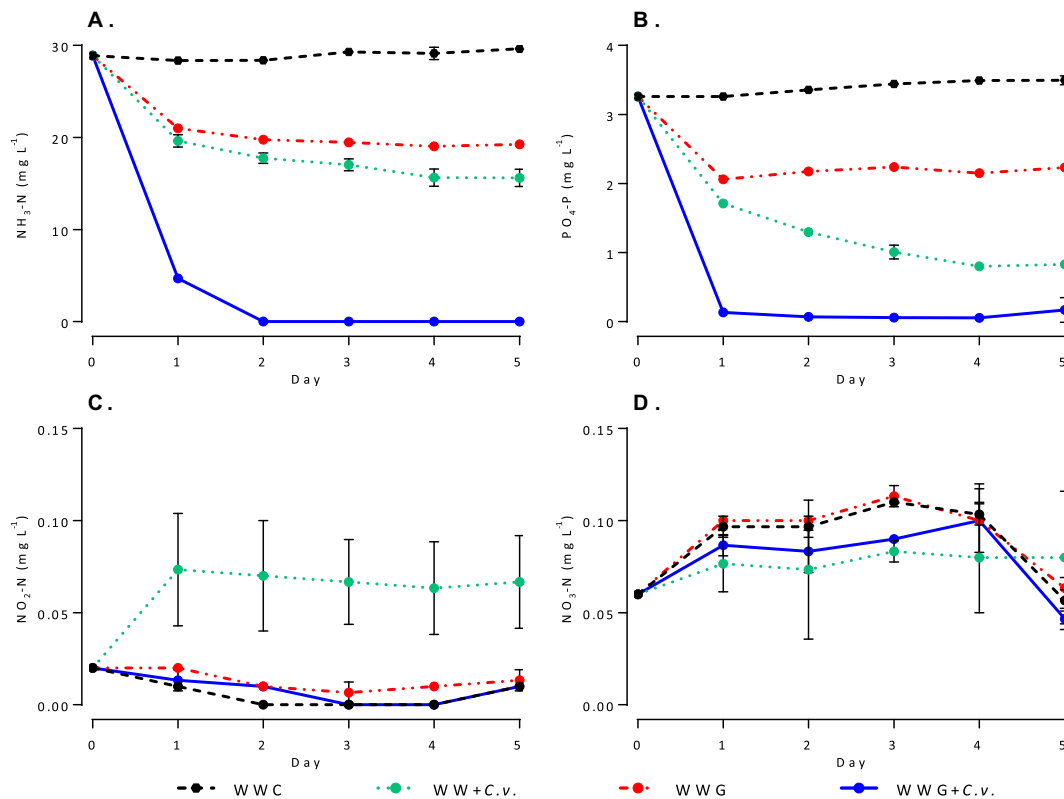
Bioavailable organic carbon, in the form of glucose, had a strong influence on the ability of *C. vulgaris* to remove inorganic nutrients from the PSW. In the case of NH<sub>3</sub>-N, this was the most abundant form of nitrogen available to the microalga in the PSW (Fig. 1A), and its removal was more effective in wastewater that was enriched with glucose compared to the untreated (no glucose) control. In the WWG + C.v treatment, NH<sub>3</sub>-N concentration rapidly declined from an initial concentration of 28.9 mg L<sup>-1</sup> to 4.6 mg L<sup>-1</sup> at day 1, and reached 0.1 mg L<sup>-1</sup> at day 2. Conversely, in the WW + C.v treatment without enrichment with glucose, concentrations of NH<sub>3</sub>-N decreased at a slower rate, reaching 19.6 mg L<sup>-1</sup> at day 1, after which only a total of 2.1 mg NH<sub>3</sub>-N was further removed over the remaining four days. In the treatments without the microalgae, NH<sub>3</sub>-N decreased to no > 19.2 mg L<sup>-1</sup> in the WWG treatment, and no reduction was recorded in the WWC treatment.

It can therefore be argued that the marked reduction in NH<sub>3</sub>-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

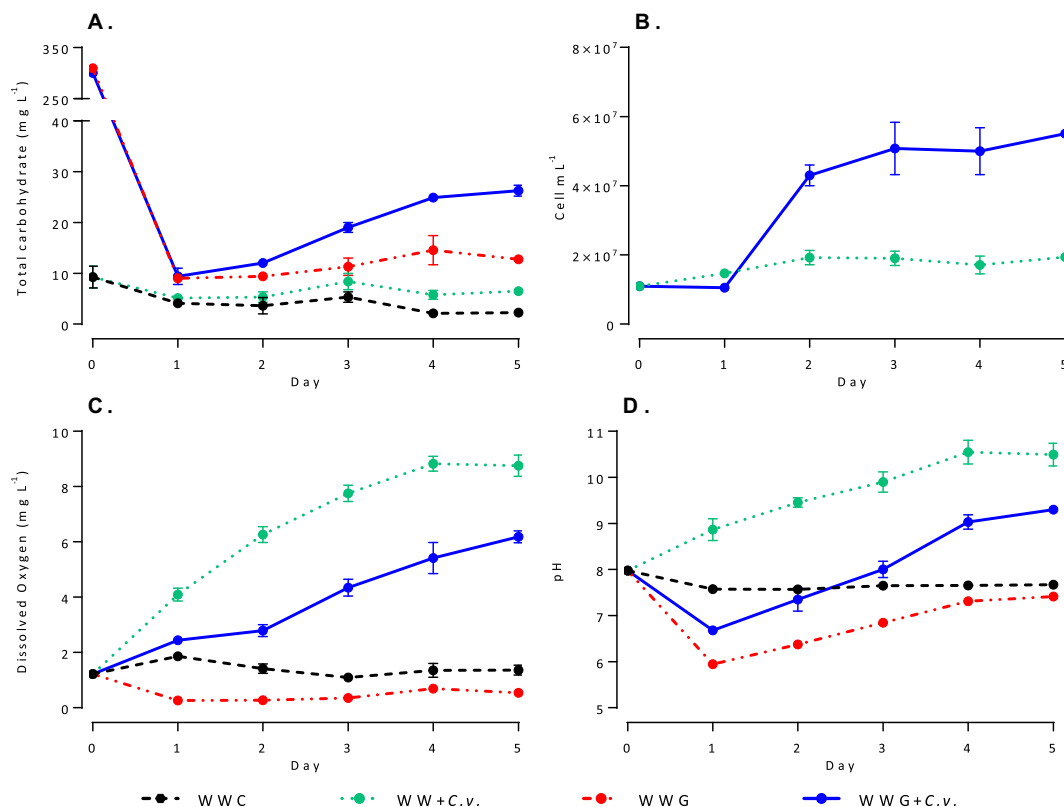
keto-acids and energy from carbon metabolism in the form of ATP and NADPH [24]. The assimilation and incorporation of ammonium into amino acids is brought about by the evolutionary conserved enzymes glutamine synthetase (GS) and glutamine 2-oxoglutarate amino transferase (GOGAT) [25]. GS fixes ammonium on a glutamate molecule to yield glutamine, and the added amino group then can act as the nitrogen donor to 2-oxoglutarate in the reduction-dependent conversion to yield two glutamate compounds catalysed by GOGAT. Further amino acid synthesis uses the carbon compound oxaloacetate in the interconversion of amino nitrogen from glutamate to yield aspartate by aspartate aminotransferase. By this mechanism, the incorporation of ammonium has been shown to increase the demand for tricarboxylic acid cycle (TCA) intermediates in microalgae, with 2-oxoglutarate and oxaloacetate being the main metabolites [26]. This demand for carbon, which feeds into the TCA cycle, can be fixed or assimilated through autotrophic or heterotrophic pathways, respectively, of mixotrophic algae like *C. vulgaris*. Therefore, when compared to the other treatments, the significantly higher NH<sub>3</sub>-N removal efficiency observed in the WWG + C.v treatment can be attributed to higher availability of bioavailable carbon, mainly to *C. vulgaris*, herein in the form of glucose ( $H(3) = 10.421, p = 0.002$  at day 1).

In wastewater treatment, NH<sub>3</sub>-N reduction also occurs through its conversion to NO<sub>2</sub>, then into NO<sub>3</sub>, and N<sub>2</sub> by nitrification and denitrification respectively. Both the NO<sub>2</sub>-N and NO<sub>3</sub>-N concentrations were consistently on the border of the detection limit in all the PSW samples from the commencement and duration of these experiments (Fig. 1C and D). We did not analyse for N<sub>2</sub>, so the process of nitrification and denitrification cannot be ruled out from occurring here. However, the likelihood of inorganic nitrogen being removed through its conversion to N<sub>2</sub> will have been limited by various chemical and physical factors associated with the treatments, albeit independently from each other. For all treatments the main limitation will have been the relatively short duration of our experiments (5 days), which was insufficient to allow for a longer generation time needed by nitrifying bacteria in PSW. Additionally the observed pH changes, inorganic carbon and O<sub>2</sub> availability in the treatments (see below) may also have limited these pathways [27]. Furthermore, the removal of NH<sub>3</sub>-N to almost below detection limits in the WWG + C.v treatment occurred within only 2 days, and likely well before nitrification had a chance to begin. The pH increase in the WW + C.v treatment and low inorganic carbon availability will have limited the formation of NO<sub>2</sub>-N [28]. Although a small increase in NO<sub>2</sub>-N was detected in this treatment (i.e. from 0.02 mg L<sup>-1</sup> to 0.07 mg L<sup>-1</sup>), this did not coincide with an equivalent amount of NH<sub>3</sub>-N removed over the 5-day duration, indicating that nitrification was not the dominant pathway in reducing the ammonium-nitrogen from the PSW. Inorganic nitrogen concentrations in the control treatments (WWC and WWG) remained fairly constant over the 5-day duration of these experiments, with the exception of NH<sub>3</sub>-N showing a slight reduction within the first day in the WWG treatment, but which was not significant ( $H(3) = 10.421, p = 0.307$  at day 1). This reduction can be ascribed to a high metabolic activity of the microbial community present in the PSW as a result of the exogenous glucose, which coincided with a decrease in total carbohydrate concentration (Fig. 2A). A major limitation to these control treatments was the low concentration of dissolved O<sub>2</sub>, which can be attributed to the cultures having been incubated statically (Fig. 2C). This will have impacted on the metabolic activity of the endogenous microorganisms in digesting and assimilating inorganic nitrogen compounds or converting them by nitrification and, thus, limiting their removal.

PO<sub>4</sub>-P was drastically reduced in WWG + C.v from 3.2 mg L<sup>-1</sup> to 0.1 mg L<sup>-1</sup> at day 1 and remained at this concentration until the end of the treatment period (Fig. 1B) ( $H(3) = 10.385, p = 0.002$  at day 1). This was a maximal removal efficiency of 96% within a period of 1 day. Notably, this is a far higher recorded rate than reported in previous studies using PSW which had reported removal efficiencies of < 50%



**Fig. 1.** Changes in the PSW concentrations for NH<sub>3</sub>-N (A), PO<sub>4</sub>-P (B), NO<sub>2</sub>-N (C) and NO<sub>3</sub>-N (D) in mg L<sup>-1</sup> treated with and without *C. vulgaris*, enriched with or without glucose. Each data point is the mean ± SD, n = 3. Some error bars are smaller than the symbol. Treatment WWC (wastewater only); Treatment WW + *C.v* (wastewater with *C. vulgaris*); Treatment WWG (wastewater with glucose); Treatment WWG + *C.v* (wastewater with glucose and *C. vulgaris*).



**Fig. 2.** Comparison of growth in *Chlorella* (B) used to bioremediate PSW enriched with or without glucose, and changes in total carbohydrate (A), dissolved oxygen in mg L<sup>-1</sup> (C) and pH (D) for each treatment for the duration of the experiment. Data points are mean ± SD, n = 3. The treatment acronyms WWC, WW + *C.v*, WWG and WWG + *C.v* are as for Fig. 1.

for the same retention time [29–31]. The efficiency of P removal is affected by both abiotic and biotic factors. In pH environments of approximately 9 or above, for example,  $\text{PO}_4^{3-}$  precipitates as a result of chemically reacting with cations in solution, mostly magnesium and calcium ions [32]. The precise efficiency of this phenomenon is dependent on the phosphorus and cation concentration, as well as temperature [32]. In regards to biotic influences, Beuckels et al. (2015) described the assimilation of P into microalgal biomass as dependent on the supply of N [33]. Their study identified that biomass P concentrations were low when the N concentration in the biomass was low because they were grown on N-limited medium, irrespective of the amount of P in the medium. Microalgae have also been reported to assimilate and store phosphorus in a mechanism referred to as ‘luxury uptake’, which occurs when phosphorus uptake exceeds the metabolic requirements of the microalgae [34]. Given the high removal efficiency of  $\text{NH}_3\text{-N}$  under neutral pH in the WWG + *C.v* treatment (Figs. 1A and 2D) and exponential growth of *C. vulgaris* (Fig. 2B), it can be inferred that the main mechanism for  $\text{PO}_4\text{-P}$  removal was through assimilation by *C. vulgaris* and other microorganisms, such as bacteria, present in the wastewater and/or associated with the microalga mainly for direct metabolic use. In comparison,  $\text{PO}_4\text{-P}$  removal in the WW + *C.v* treatment was a result of its assimilation initially and subsequent precipitation after day 1 because of a gradual increase in the pH above 9 (Fig. 2D). Here,  $\text{PO}_4\text{-P}$  concentrations decreased from  $3.2 \text{ mg L}^{-1}$  to  $1.7 \text{ mg L}^{-1}$  by day 1, and then continued to decrease reaching minimal concentrations of  $0.8 \text{ mg L}^{-1}$  by day 4. The low removal and consequently assimilation rate of  $\text{NH}_3\text{-N}$  by *C. vulgaris* will have likely influenced the internal N concentration of the microalgae, thus also affecting the assimilation of P in this treatment. However, the continuous removal of phosphorus by the microalgae through luxury uptake after day 1 in the WW + *C.v* treatment cannot be ruled out (Fig. 2B). This same trend of a slow decrease in  $\text{PO}_4\text{-P}$  after day 1 was not observed in the WWG treatment despite displaying a similar reduction in  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$  as in the WW + *C.v* treatment. The reduction in  $\text{PO}_4\text{-P}$  concentration in the WWG treatment by day 1 was likely through its assimilation and incorporation by the indigenous microbial community present in the PSW, concurrent with the reduction of  $\text{NH}_3\text{-N}$ . As anoxic conditions developed in the control treatments, aerobic metabolism and degradation of the inorganic compounds will have slowed (Fig. 2C). However, as the pH did not increase above 8 in these treatments, no substantial decrease in  $\text{PO}_4\text{-P}$  could be attributed to phosphate precipitation.

Comparing the capacity to remove inorganic nitrogen and phosphorus between the treatments, the results indicate that regardless of the treatment condition, with or without enrichment, the microalgae were mainly responsible for its elimination from the PSW. In the control treatments (without microalgae) the most effective decline in  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$  was in the WWG treatment, while WWC exhibited no noteworthy change from the initial concentrations of the PSW. This suggests that the natural microbial community of the PSW alone was not able to effectively remove or convert the inorganic compounds to any great extent under the culture conditions imposed. Although the influence of the microbial community cannot be completely disregarded, with respect to eliminating the inorganic N and P their ability to directly do so is limited. This finding is consistent with previous studies employing microalgae-bacteria co-cultures. For example, Su et al. (2012) investigated the potential of a co-culture composed of wastewater-born algae consortium (majority filamentous blue-green algae) and activated sludge, inoculated at different ratios (w/w) on nutrients removed from pre-treated wastewater [35]. The removal efficiencies of total Kjeldahl nitrogen and  $\text{PO}_4\text{-P}$  removal at day 10 were respectively 95.5% and 93.5% in the 5:1 algae-bacteria co-culture, whereas in the reactor with only sludge the concentrations declined to 31.4% and < 10% respectively. Ma et al. (2014) directly examined the influence of bacteria removing nutrients from concentrate, a waste stream following sludge dewatering, with *C. vulgaris* by varying the initial

concentration of bacteria in the co-culture [36]. Their results revealed no significant difference in nutrient removal from the wastewater with increasing bacteria concentrations, implying that the presence of bacteria had little effects on the removal of the inorganic compounds, at least within the investigated range. In the present study, the contribution of the bacteria in the microalgae treatments to remove the inorganic N and P may have been limited by the composition of the microbial community and environment of the treatment. Biological nutrient removal from wastewater is dependent on specific microorganisms (i.e. nitrifying, denitrifying and phosphorus accumulating organisms), which are encouraged to grow and function by cycling the wastewater through anaerobic, aerobic and anoxic environments [1,27]. The presence of these microorganisms are naturally low in influent wastewater, inhibited by the high concentration of carbonaceous-BOD in influent and settled wastewater, a situation that would have been exacerbated by the deliberate organic carbon enrichment carried out in the experiments reported here. Without these specific microorganisms the removal of N and P in wastewater treatment tends to be minimal. It can be suggested that the microbial population in the microalgae treatments was not composed of these appropriate or adapted microorganisms to facilitate the N and P removal beyond their metabolic capabilities. Another aspect that may have limited the microbial population in removal of inorganic N and P in the microalgae treatments is the high pH environment, particularly in the WW + *C.v* treatment (Fig. 2D). Elevated pH (discussed below) in conjunction with high dissolved oxygen concentration (Fig. 2C) in a light environment mediate photo-oxidative destruction of coliform bacteria [37,38].

### 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]. Fig. 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  $9.2 \text{ mg L}^{-1}$ , which was lower than the theoretical range of 50 to  $120 \text{ mg L}^{-1}$  for municipal wastewater, as suggested by Gray (2004). The TC concentration in the WW + *C.v* and WWC treatments declined only slightly to  $4.6 \text{ mg L}^{-1}$  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  $305.1 \text{ mg L}^{-1}$  to  $9.2 \text{ mg L}^{-1}$  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  $138.3 \text{ mg L}^{-1} \text{ O}_2$  and  $133.6 \text{ mg L}^{-1} \text{ O}_2$ , 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

Table 1

Chemical Oxygen Demand (soluble) concentrations for PSW in the four treatments in the initial glucose enriched experiment. Values are mean  $\pm$  SD,  $n = 3$  reported as  $\text{mg L}^{-1} \text{ O}_2$  for the initial composition of PSW with exogenous glucose and for the final readings taken on day 5.

Condition	Initial $\text{COD}_s$	Final $\text{COD}_s$
WW	$141.9 \pm 4.2$	$101.6 \pm 5.6$
WWG	$416.3 \pm 15$	$138.3 \pm 3.1$
WW + <i>C.v</i>	$141.9 \pm 4.2$	$106.6 \pm 8.4$
WWG + <i>C.v</i>	$422.4 \pm 5.8$	$133.6 \pm 9.1$

conditions.

Interestingly, the beginning of the *C. vulgaris* stationary growth phase at day 2 in the WWG + *C.v* (Fig. 2B) coincided with an increase in TC concentrations (Fig. 2A). Henderson et al., (2008) reported an increased production of dissolved organic carbon during the stationary growth phase for various microalgal species, and this was attributed to the excretion of extracellular polysaccharide substances (EPS) by the microalgae [40]. Hence, the observed increase in TC concentrations after day 2 in the WWG + *C.v* treatment could be attributed to EPS production during the stationary phase [41].

### 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 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*) (Fig. 2B). Although cell counts 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<sup>-1</sup> and 42.8 mg L<sup>-1</sup> for the treatments respectively. The WWG + *C.v* treatment had the highest biomass yield with 419.1 mg L<sup>-1</sup> compared to 111.7 mg L<sup>-1</sup> 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 (Fig. 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<sub>2</sub> by RuBisCO, which is converted from HCO<sub>3</sub><sup>-</sup>. This photosynthetic-driven process leaves OH<sup>-</sup> ions in the cell which have to be neutralised with H<sup>+</sup> 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<sub>2</sub> to bicarbonate ratio, and eventually a reduced absolute CO<sub>2</sub> concentration. As we employed a static culture system, the contribution of atmospheric CO<sub>2</sub> will have been negligible.

Furthermore, the unfavourable (high pH) environment present may also have limited the growth of other members of the microbial community in the PSW and thus reduced their production of CO<sub>2</sub> via respiration that would have otherwise served *C. vulgaris* with an alternative source of this essential compound for photosynthesis. Additionally, the pH rise in the WW + *C.v* treatment will have had a strong influence on its NH<sub>3</sub>-N removal efficiency (Fig. 1A). While

ammonium (NH<sub>4</sub><sup>+</sup>) is the preferred inorganic nitrogen source for microalgae, a rise in pH above 8 leads to its dissociation to form free ammonia (NH<sub>3</sub>) which is toxic to microalgae and other aquatic organisms [43]. The pH in this treatment increased from 7.97 to 10.49 at a relatively constant rate over the 5-day duration of these experiments (Fig. 2D). This will have contributed to the formation of free ammonia creating an unfavourable environment for nutrient assimilation and microalgae growth. The alkalisation also suggests a reduction and consequent limitation in inorganic carbon because of its ability to buffer pH changes in the medium environment. The resultant drop in NH<sub>3</sub>-N removal after day 1 in the treatment supports the lack of available carbon before the onset of ammonia toxicity, most likely because of the low inorganic carbon to the microalgae will have limited the assimilation of NH<sub>3</sub>-N, as described above (Section 3.1.1).

Conversely, the pH in the glucose-enriched treatments decreased rapidly within the first day to below 6.6 for WWG + *C.v* and 5.9 for WWG (Fig. 2D). This drop in pH coincided with the removal of the added glucose in both treatments (Fig. 2A), suggesting that acidification of the PSW did not negatively affect the consumption of this substrate. The anoxic environment in the WWG treatment (Fig. 2C) will have driven the degradation of organic compounds, including glucose, to produce organic acids through the process of acidogenesis and acetogenesis and thus the observed pH reduction in this treatment [27]. It should also be noted that the pronounced removal of NH<sub>3</sub>-N and PO<sub>4</sub>-P will have also influenced the overall extracellular H<sup>+</sup> concentration and thus influencing the observed shifts in the pH.

### 3.2. Treatment reproducibility assessed across environmental samples and alternative carbon sources

The small-scale treatment of PSW with exogenously added glucose was used to evaluate the growth of *C. vulgaris*, its removal of inorganic compounds, and to analyse for other biochemical and physical changes under the different treatment regimens evaluated. This provided a useful understanding of the treatment performance under static culturing conditions revealing that it was limited, either because of the limited bioavailability of carbon to the microalga or detrimental effects from pH changes. In order to upscale this into a commercially-viable system, we would need to demonstrate that this process can be consistently replicated with PSW collected at any time to take into consideration biotic and abiotic variability of the wastewater throughout the year. To investigate this, a further three batches of PSW were collected and treated separately and sequentially with *C. vulgaris* employing the same static culturing approach as described and evaluated above. In addition to enriching with glucose, treatments with glycerol and CO<sub>2</sub> were also included to compare between the use of a different organic and inorganic carbon source.

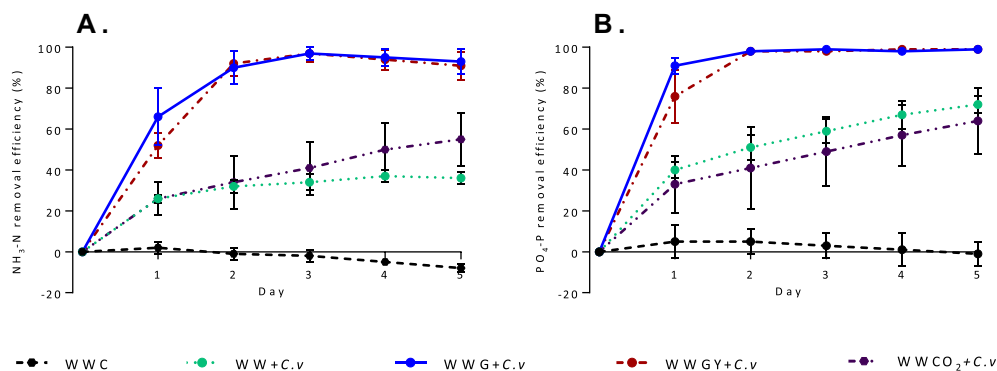


Fig. 3. Percentage removal efficiency of NH<sub>3</sub>-N (A) and PO<sub>4</sub>-P (B) averaged from the three batches of PSW treated with and without *C. vulgaris*, enriched or not with either glucose, glycerol or CO<sub>2</sub>. Data points are mean ± SD, n = 3 (1 for each batch of PSW). Treatment WWC (wastewater only); Treatment WW + *C.v* (wastewater with *C. vulgaris*); Treatment WWG + *C.v* (wastewater with glucose and *C. vulgaris*); Treatment WWGY + *C.v* (wastewater with glycerol and *C. vulgaris*); Treatment WWCO<sub>2</sub> + *C.v* (wastewater with CO<sub>2</sub> and *C. vulgaris*).



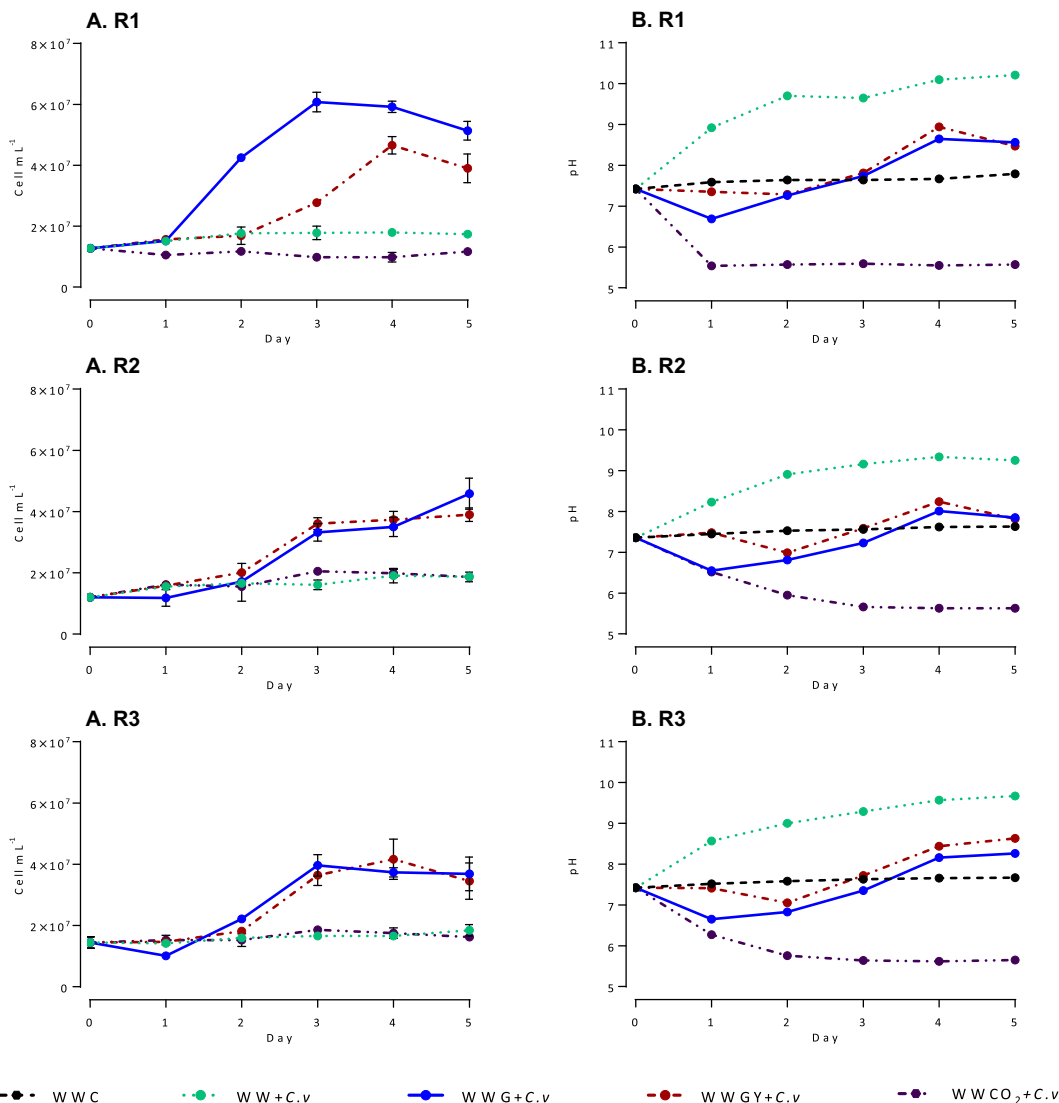


Fig. 4. Cell concentration ( $\text{cell mL}^{-1}$ ) (A) and pH (B) for each PSW batches treated under the conditions with and without *C. vulgaris*, enriched or not with either glucose, glycerol or CO<sub>2</sub>. Cell concentration is an average of three counts (pseudo replicate for each batch of PSW) and pH from one measurement from each treatment. R1, R2 and R3 correspond to PSW batch sample 1, 2 and 3 respectively.

Fig. 3 shows the average percentage removal efficiency for NH<sub>3</sub>-N and PO<sub>4</sub>-P for each treatment from the three batches of PSW combined. Overall, the efficiency in NH<sub>3</sub>-N and PO<sub>4</sub>-P removal across the batches of PSW was effective and reliable in the treatments with exogenous organic carbon. The treatments enriched with glucose and glycerol performed the same with respect to their removal of NH<sub>3</sub>-N and PO<sub>4</sub>-P, with a respective 91% and 98% average efficiency in both treatments (both  $p < 0.01$  at day 2). In comparison, WWCO<sub>2</sub> + *C.v* had an average removal efficiency of 55% for NH<sub>3</sub>-N and 64% for PO<sub>4</sub>-P. The acidification of the medium in the WWCO<sub>2</sub> + *C.v* treatment is the most likely reason that caused the decreased removal efficiency in NH<sub>3</sub>-N and PO<sub>4</sub>-P compared to the organic carbon enriched treatments (Fig. 4B, R1 – R3). Despite the limited sparging of CO<sub>2</sub>, the aqueous dissolved CO<sub>2</sub> in this treatment resulted in a pH drop to approximately 5.5 after day 2, which may have adversely affected growth of the microalga. The presence of excess CO<sub>2</sub> available to the microalga was to enhance photosynthetic productivity. However, microalgal growth itself was limited in this treatment showing a similar growth pattern and cell concentration as in the WW + *C.v* treatment which had no form of enrichment (Fig. 4A R1 – R3). It has been suggested that excess CO<sub>2</sub> concentrations can lower or inhibit microalgae respiration because

of its strong influence on photosynthetic efficiency [44]. This may, hence, explain the observed lower growth in this treatment condition. Similarly, NO<sub>2</sub>-N and NO<sub>3</sub>-N concentrations between the treatment types showed no substantial or detectable change (Fig. S1). However, small differences in the initial concentration between the PSW batches of these inorganic nitrogen compounds was recorded, although this seemed to have little effect on the overall performance of the process.

The final effluent concentrations from a wastewater treatment system are a key criteria in validating the performance of the process. Meeting final discharge maximums set at the more restrictive limit of 10 mg L<sup>-1</sup> TN, 1 mg L<sup>-1</sup> TP and 125 mg L<sup>-1</sup> COD laid out by the Urban Wastewater Treatment Directive are preferable [5]. Although the organic carbon-enriched treatments removed an average of > 90% of NH<sub>3</sub>-N and PO<sub>4</sub>-P, between the three batches of PSW that were treated, the variation in the initial concentration of these inorganic compounds in each PSW batch influences the efficiency of their removal. Batch 2 and 3 had the highest concentration of TN compared to batch 1 (Table 2). This impacted on the final TN effluent concentration, as a higher initial concentration led to a higher final concentration (Fig. 5 and Table S3). For batch 3, final TN was > 11 mg L<sup>-1</sup> in both the glucose and glycerol enriched treatments, and COD > 125 mg L<sup>-1</sup> O<sub>2</sub>

**Table 2**

Physiochemical characteristics of the three batches of PSW used in the experiment to validate the reproducibility of the static treatment process, analysis from centrifuged samples. Concentrations recorded in  $\text{mg L}^{-1}$ .

	$\text{NH}_3\text{-N}$	$\text{PO}_4\text{-P}$	$\text{NO}_2\text{-N}$	$\text{NO}_3\text{-N}$	$\text{COD}_s$	pH	TN
Batch 1	$23.4 \pm 0.2$	$2.9 \pm 0.1$	$0.30 \pm 0.0$	$0.41 \pm 0.0$	$113.9 \pm 5.3$	7.42	$29.8 \pm 0.2$
Batch 2	$34.9 \pm 0.5$	$4.3 \pm 0.3$	$0.03 \pm 0.0$	$0.06 \pm 0.0$	$219.6 \pm 10.0$	7.36	$38.7 \pm 1.8$
Batch 3	$34.7 \pm 0.2$	$3.7 \pm 0.1$	$0.03 \pm 0.0$	$0.06 \pm 0.0$	$182.0 \pm 6.1$	7.42	$44.5 \pm 0.7$

in the glucose enriched treatment. This suggests that there is a limitation between the maximum N concentrations that could be treated in the presence of the enriched carbon quantity added to the PSW batches in this study. To explore this further, an experiment with PSW and the concentration of organic carbon used throughout this study with controlled N ratios would need to be carried out under static culturing to further validate this effect. The maximum microalgal cell concentrations reached were also affected, which were lower in batches 2 and 3 (Fig. 4A, R2 and R3). *C. vulgaris* increased to  $> 4.5 \times 10^7$  cells  $\text{mL}^{-1}$  in batch 1, with a maximum cell concentration of  $6.08 \times 10^7$  and  $4.65 \times 10^7$  cells  $\text{mL}^{-1}$  for the treatments enriched with glucose and glycerol, respectively. In batches 2 and 3, the maximum cell concentration reached in either of these organic carbon enrichment treatments was  $< 4.5 \times 10^7$  cells  $\text{mL}^{-1}$ .

Future work could evaluate an alternative source of organic carbon to determine its impact on PSW treatment with *C. vulgaris* under the static co-culture treatment process used from laboratory setting to commercial application. Despite the low quantities of glucose or glycerol used this is not cost effective at a commercial scale, so therefore, an alternative organic carbon source(s) ideally from a waste source is needed to substitute for their use [45]. Optimisation of the process to mitigate the fluctuations in pH could also be explored and potentially easily overcome with the use of an appropriate photobioreactor design, preferably incorporating a semi-continuous treatment process.

#### 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  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$  in the WWG + *C.v* treatment, from a concentration of 28.9 to 0.1  $\text{mg L}^{-1}$  and 3.2 to 0.1  $\text{mg L}^{-1}$  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  $\text{NO}_3\text{-N}$  and  $\text{NO}_2\text{-N}$  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  $\text{CO}_2$ . For all PSW batches, organic carbon enrichment with *C. vulgaris* resulted in a consistent rate of reduction ( $> 90\%$ ) of  $\text{NH}_3\text{-N}$  and  $\text{PO}_4\text{-P}$ , 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,  $\text{NH}_3\text{-N}$ ,  $\text{PO}_4\text{-P}$  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

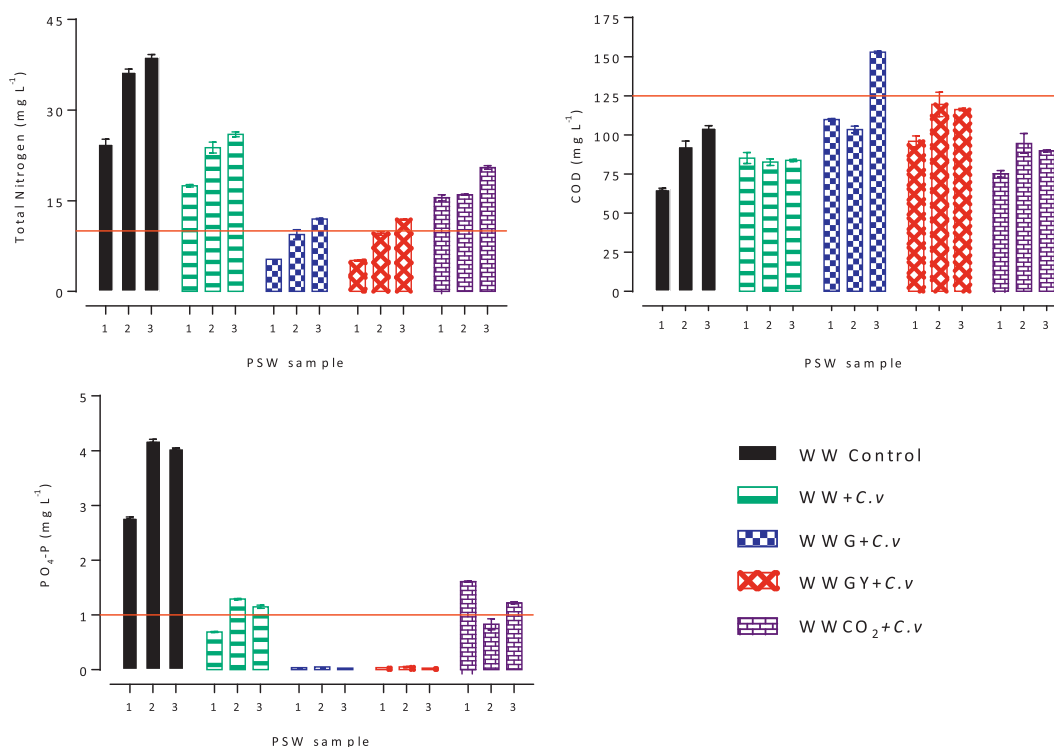


Fig. 5. Final effluent characteristics for each of the separate PSW batches are presented with 1, 2 and 3 corresponding to the separate batch samples. Red lines indicate the stricter discharge limits permissible by EU law [5]. The 1  $\text{mg L}^{-1}$  limit for  $\text{PO}_4\text{-P}$  does not represent the true limit as this is set for TP which was not analysed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of *C. vulgaris* to treat PSW without aeration offers a key area to develop low energy biological wastewater treatment compared to conventional secondary processes.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.algal.2017.04.011>.

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