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Journal of Cleaner Production 327 (2021) 129436

Contents lists available at ScienceDirect

Journal of Cleaner Production

journal homepage: www.elsevier.com/locate/jclepro

Effect of pot-ale enrichment on the treatment efficiency of primary settled wastewater by the microalga *Chlorella vulgaris*

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ARTICLE INFO

Handling editor: Cecilia Maria Villas Bôas de Almeid

Keywords:
Microalgae
Wastewater treatment
Pot ale
Organic waste
Bioremediation
Photobioreactor

ABSTRACT

This study evaluated the performance of microalgae under static cultivation for primary settled municipal wastewater (PSW) treatment as a low energy treatment process. The availability of a suitable carbon substrate was determined to be the main limiting factor affecting the algal treatment performance. To overcome the material cost of applying commercial sources of organic carbon, we evaluated pot ale – a carbohydrate-rich by-product from the production of malt whiskey – as a carbon substrate to promote microalgae growth and the removal of nitrogen (NH_3–N) and phosphate (PO_4–P) in PSW. For this, the mixotrophic microalgal species *Chlorella vulgaris* was used in batch experiments of PSW enriched with pot ale. Characterisation of the wastewater in the microalgae treatments compared with the control treatments (WWC) and wastewater with pot ale (WWPA) highlighted that *C. vulgaris* was a key organism in the algal-bacterial consortium responsible in inorganic N and P removal. We also observed a high variability in the characteristics of PSW across independent batches enriched with pot ale, which resulted in variability in the N and P removal efficiency by the alga, from 99% to 58% at reducing NH_3–N, and from 94% to 58% at reducing PO_4–P. As an extension of these batch-wise operated treatments, we investigated removal of NH_3–N and PO_4–P under semi-continuous operation with pot ale enrichment and found this to be a viable system for potential further development. This work highlights the use of pot ale enrichment with microalgae as a promising application for enhancing the efficiency of inorganic nutrient removal from PSW.

1. Introduction

Treating municipal wastewater is necessary to limit the impact that carbonaceous, nitrogenous and phosphorus-containing matter present in spent water can have on receiving aquatic systems. Conventional wastewater treatment systems employing the activated sludge or biological nutrient removal process as the main phase of treatment demonstrate a high proficiency at removing these contaminants. However, these processes are described as problem shifting, as they are marred with causing secondary pollution because of high energy consumption and the production of waste sludge and greenhouse gases (Evans et al., 2017). To improve the environmental impact of wastewater treatment, considering stricter effluent discharge standards in particular, treatment processes that have low energy consumption without affecting performance are needed. In this respect, a potential and more sustainable biological treatment process for the efficient removal of inorganic and organic nitrogen (N) and phosphorus (P) from wastewater is using microalgae. Although this concept has been extensively researched, limited commercial development and implementation of microalgae to treat wastewater has been achieved, due largely to cost and energy burdens.

Microalgae acquire the majority of their carbon via photosynthetic carbon fixation in which inorganic carbon is incorporated into organic carbon substrates (Falkowski and Raven, 2007). However, a number of microalgae exhibit facultative heterotroph capabilities, consuming organic carbon substrates over CO_2 fixation (Perez-Garcia et al., 2011). Alternatively, certain microalgal species are mixotrophic in which both

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https://doi.org/10.1016/j.jclepro.2021.129436
Received 12 June 2021; Received in revised form 2 October 2021; Accepted 19 October 2021
Available online 25 October 2021

Footnotes

BBM	Bold basal media
COD	Chemical oxygen demand
N	Nitrogen
NH_3–N	Ammonia-Nitrogen
NO_2–N	Nitrite Nitrogen
NO_3–N	Nitrate-Nitrogen
P	Phosphorus
P#	Pot ale batch number
PO_4–P	Phosphate-Phosphorus
PSW	Primary settled wastewater
R#	Wastewater batch number
SC	Semi-continuous
TCA	Tricarboxylic acid cycle
TP	Total phosphate
WW + *C.v*	Wastewater with *C. vulgaris*
WWC	Wastewater control
WWPA	Wastewater with pot ale
WWPA + *C.v*	Wastewater with pot ale and *C. vulgaris*

photoautotrophic and heterotrophic carbon assimilation and metabolism occur simultaneously (Perez-Garcia et al., 2011; Cheirsilp and Torpee, 2012). In the presence of a suitable organic carbon source, the synergistic effect of the two processes has been shown to enhance microalgal productivity. The effects of other organic carbon sources, including glycerol, fructose or sodium acetate have also been studied in mixotrophic cultivation of freshwater microalgae (Mohsenpour et al., 2020). The influence of the organic carbon source on microalgae productivity varies not only between organic carbon sources, but also the concentrations present in the medium and even among different microalgae species cultured with the same carbon source (Liang et al., 2009; Sforza et al., 2012; Zhan et al., 2017). Various organic carbon by-products generated from manufacturing processes have been successfully proven to support microalgal growth under heterotrophic or mixotrophic conditions. For instance, sugar cane juice (Cheng et al., 2009), cassava starch hydrolysate (Wei et al., 2009), corn powder hydrolysate (Gélinas, 2015), dairy (Abreu et al., 2012) and brewery waste (Lutzu et al., 2015), amongst others, have proven useful in this respect. However, the main focus of these studies was to improve microalgal biomass and lipid yield. An alternative opportunity, thus, arises to supplement PSW with a carbon-rich by-product as a relatively inexpensive, and if possible, also sustainable, source to enhance the treatment efficiency of microalgae for the efficient removal of N and P.

A carbon-rich by-product for potentially enhancing microalgae wastewater treatment efficiency is pot ale, a residue remaining in the pot sill after the first distillation step in whisky production (Traub, 2015; White et al., 2016). Characterised as an acidic (pH $<$ 4) brown-red turbid liquid, pot ale is mainly composed of yeast and barley fractions that are present in both the solid and soluble phase. Presently, the disposal of pot ale is a concern as its high chemical oxygen demand (COD), N and P content are associated with expensive treatment processes (Mohana et al., 2009; Pant and Adholeya, 2007). Conventional treatment of pot ale is achieved through anaerobic digestion with the co-generation of methane, followed by phosphate precipitation and biological nitrification and denitrification (Mohana et al., 2009). Despite the high depurative efficiency achieved (<90%), effluents of pot ale still retain high organic loads with COD concentrations around 10 g L^{-1} O_2. It should be noted that the inherent variation in its composition following the distillation process makes stable anaerobic digestion difficult to maintain (Mohana et al., 2009; White et al., 2020). In some instances, the methane produced is contaminated with hydrogen sulphide, with concentrations reaching as high as 2 g L^{-1}, making it an unsuitable product without further processing. With production at an estimated 8 L of pot ale per litre of alcohol, accounting for approximately 2–3 million tonnes of pot ale generated annually in Scotland alone, it presents a substantial source of a carbon-rich by-product (Traub, 2015; White et al., 2016). With the need for a more cost-effective and sustainable disposal process, the coupling of pot ale with PSW treatment by microalgae is a highly promising solution.

In a recent study, we demonstrated that organic carbon enrichment of PSW improved the treatment efficacy by the mixotrophic microalga, *C. vulgaris*, under static culture conditions (Evans et al., 2017). Although using glucose or glycerol as organic substrates is suitable for research in a laboratory setting, from a commercial perspective these substrates entail a high cost when required for industrial and large-scale applications, as would be in wastewater treatment. Consequently, it is imperative that alternative, low-cost organic carbon substrates are identified and assessed for their applicability, ideally from a waste source, and pot ale falls perfectly within these criteria. Here, in this study we evaluated the effect that enrichment of PSW with pot ale, as an organic carbon substrate amendment, has on a static and then subsequently semi-continuous microalgal treatment process, in order to investigate its potential for development to an industrial scale.

2. Materials and methods

2.1. Microalgae strain, medium and maintenance

The algae strain *Chlorella vulgaris* CCAP 211/79 was cultivated in a modified Bold basal medium (BBM) and used in this study. The strain was originally isolated from a waste solvent biofilter at Heriot-Watt University, Edinburgh, UK. For all experiments, a seed culture was grown 7 days prior to use as the inoculum into the wastewater, at which point the algae were in an approximate late exponential phase of growth. For culturing, 500 mL screw-capped Schott glass bottles, containing 350 mL BBM, were inoculated and the bottles aerated continuously with filtered (0.22 μm) atmospheric air at a flow rate of 0.15 of air volume per volume of liquid per minute (In-Line HEPA filter, Whatman International Ltd., UK). Incubation conditions (15 ± 1 °C; 12:12 light-dark cycle; photon flux of 100 μmol m^{-2} s^{-1} (US-SQS/L probe, Walz, Germany) were the same for the stock cultures, seed cultures and the experimental runs.

2.2. Wastewater and pot ale sources

The municipal wastewater was collected from the primary settling effluent channel at the Seafield Wastewater Treatment Plant, Edinburgh, UK. The only processing that was applied to the wastewater included filtration of each wastewater sample through a Whatman 113 filter (pore size 30 μm, Whatman International Ltd., UK) to apply a degree of consistence in the turbidity between samples (unless indicated otherwise). No sterilisation or further processing was applied to the collected wastewater.

Samples of pot ale used in this study were previously subjected to a protein extraction process that removed approximately more than 60% of the soluble protein fraction. To avoid the introduction of organisms other than the autochthonous microbial community of the wastewater and bacteria associated with the microalga, upon receipt the pot ale was filter sterilised (0.22 μm) and stored at 4 °C until used. No pH adjustment or amendments with nutrient salts were applied.

2.3. Experimental design

The following subsections describe the experimental design and setup, and the reader is also referred to the schematic shown in Fig. 1 which illustrates this.

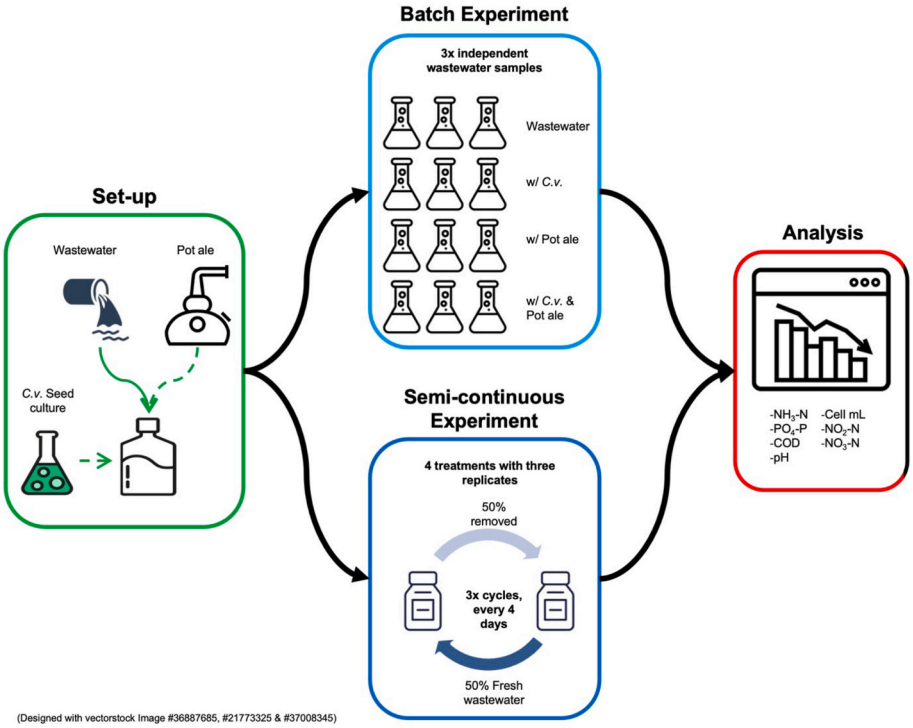

Fig. 1. Schematic of the experimental design, setup and analysis used.

2.3.1. Batch-wise treatment of pot ale enriched wastewater

For the experimental setup, three pot ale enrichment treatments were prepared (labelled P1, P2 and P3), each with different initial physicochemical (organic and inorganic) compositions (Table 1). These were prepared using three batches of collected PSW, which were labelled as R1, R2, and R3 (Table 2). Pot ale composition, as well as the initial composition of each PSW batch (with and without pot ale amendment), were analysed immediately prior to commencing the experiments by inoculation with the microalga. In total, four conditions (each in triplicate) were set up and labelled as follows: wastewater control (WWC), wastewater with pot ale (WWPA), wastewater with *C. vulgaris* (WW + *C.v*), and wastewater with pot ale and *C. vulgaris* (WWPA + *C.v*). Each treatment consisted of a working volume of 450 mL in a 500 mL glass bottle. For the preparation of each treatment, a seed culture of *C. vulgaris* grown on BBM was concentrated by centrifugation (3500×*g*; 10 min) and washed twice with the filtered (Ø 90 mm, pore size 30 μm, Whatman International Ltd., UK) wastewater collected on the day. Three litres of filtered wastewater were then transferred to a 5 L glass bottle and inoculated with the washed cells to a dry weight concentration of 0.1 g L^{-1}. From this, the WW + *C.v* treatment was aliquoted out of and into three glass bottles. The remaining 1.5 L of the wastewater with *C. vulgaris* was amended with pot ale at a set ratio of 1:150 v/v; this resulted in an equivalent COD increase between 250 and 260 mg L^{-1} O_2. In a clean bottle, this step was repeated separately without the addition of the microalgae for the enrichment of the wastewater with pot ale only treatment. This experiment was repeated a total of three times with each run treating a different sample of the collected wastewater enriched with a different batch of pot ale.

2.3.2. Semi-continuous treatment of pot ale enriched wastewater

With the exception of the wastewater not being filtered, to investigate the treatment performance under semi-continuous operation the same four treatments as described above in the batch experiment were set up in an identical manner. For this, a batch sample of each treatment was set up, and after 4 days half of the initial volume was removed and replaced with the appropriate wastewater type – wastewater only in the WWC and WW + *C.v* treatments, and pot ale enriched wastewater (without additional microalgae) in the WWPA and WWPA + *C.v* treatments. This process was repeated a total of three cycles during the operation of these semi-continuous treatments. On each day that a cycle occurred, a fresh wastewater sample was collected on that day, while the same batch of pot ale was used for the entire experiment.

2.4. Analysis of inorganics

Methods for the analysis of ammonia, nitrate, nitrite and phosphate were adapted from the Standard Methods for the Examination of Water and Wastewater (Rice et al., 2017). These methods were modified to permit the analysis of smaller volumes (5 mL instead of 25 mL) without affecting the chemistry of the reaction. Prior to analysis, all samples were centrifuged (3500×*g*; 10 min) to minimise optical interference from either the microalgal cells or particular matter. Absorbance was measured on a Genesys 20 spectrophotometer with a 1 cm light path. For each inorganic compound, a calibration graph of known concentrations

Table 1
Characterisation of three deproteinated pot ale samples from undisclosed malt whiskey distilleries; values are a mean ± SD, of n = 3 (pseudo replicates) with organic and inorganic N or P concentrations reported in mg L^{-1}, and COD concentration in mg L^{-1} O_2.

PA Batch	NH_3–N	NO_2–N	NO_3–N	TN	PO_4–P	TP	COD	pH	mS/cm
P1	<0.1	<0.02	0.26	886	436	622	43,100	3.32	4.33
P2	<0.1	0.14	0.57	696	442	634	41,400	3.30	3.74
P3	<0.1	0.03	0.31	327	334	482	40,700	3.28	4.18

NH_3–N and NO_3–N concentrations from pot ale with pH 7.

Table 2
Characterisation of each PSW batch; values are a mean ± SD, of n = 3 (pseudo replicates) with concentrations reported in mg L^{-1}.

PSW Batch	NH_3–N		PO_4–P		NO_2–N		NO_3–N		COD	
	-	+	-	+	-	+	-	+	-	+
R1	20.9	19.6	5.7	9.8	0.02	0.02	0.05	0.05	130	393
R2	47.8	46.8	5.9	9.1	0.03	0.02	0.06	0.11	191	440
R3	35.2	34.4	4.4	7.2	0.03	0.02	0.05	0.06	168	415

"-" without pot ale and "+" with pot ale.

versus their respective absorbance was plotted using commercial stock standards (Hach, UK) for each respective inorganic compound. Ammonia was determined by the phenate reaction with the concentration of NH_3–N based on the intensity of indophenol formed by the reaction of NH_3 with phenol and hypochlorite as catalysed by sodium nitroprusside (adapted from APHA 4500 – NH3.F). Nitrite was determined by the diazotization reaction in which a red-purple azo dye is formed in proportion to the amount of NO_2 present (adapted from APAH 4500 – NO_2.B). Nitrate was determined by a modified hydrazine reduction reaction in which NO_3 is reduced to NO_2 by hydrazine sulphate catalysed by copper ions (adapted from APAH 4500 – NO_3.H). Phosphate was determined by the ascorbic acid reaction with the concentration of PO_4–P based on the intensity of phosphomolybdenum blue complex formed (adapted from APAH 4500 – P.E).

2.5. Analysis for chemical oxygen demand

Chemical oxygen demand (COD) was analysed on wastewater samples filtered to 0.45 μm using the mercury-free, closed-reflux digestion process quantified by the titrimetric method, as previously described (Westwood, 2007). Following titration, the COD concentration was calculated according to the following formula:

$$COD\ as\ mg\ L^{-1}O_2 = (Vb - Vs) * DF * M * 4000$$

where, Vb and Vs respectively are the volumes of the titrant iron (II) ammonium sulphate in the blank and sample, DF is the dilution factor of the sample, and M is the molarity of iron (II) ammonium sulphate solution (0.025 M).

2.6. Microalgae growth

Cell concentrations of *C. vulgaris* in liquid were determined by direct counting using a Neubauer improved haemocytometer. Appropriately diluted samples were amended with Lugol's solution to a concentration of 0.1% (v/v) and allowed to stand for approximately 1 h prior to counting.

2.7. Statistical analysis

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. When any of the data were found not to comply with a normal distribution, a nonparametric Kruskal-Wallis test by rank was run to determine if a difference in the median concentration values of an inorganic compound occurred between the treatments at a selected time point. For statistically significant differences ($p < 0.05$), a pairwise comparison using Dunn's procedure with a Bonferroni correction for multiple comparisons was followed. Unless otherwise stated, the reported significance refers to a comparison of a treatment to the control treatment (WWC) at the time point (day) stated.

3. Results and discussion

3.1. Characterisation of pot ale

Pot ale COD concentration was consistent across all samples, with a mean concentration of 42.8 ± 1.9 g L^{-1} O_2, indicating a high oxidisable-carbon content. Inorganic analysis revealed NO_3–N to be the main inorganic N species, however, the average concentration across all the pot ale samples was low, at 0.36 ± 0.1 mg L^{-1}. Both NO_2–N and NH_3–N concentrations were found to be negligible or below the limit of quantification. To accurately determine NH_3–N and NO_3–N concentrations, an additional analysis was conducted on pot ale adjusted to pH 7, to allow an alkaline environment to form following the addition of the reagents. This was necessary to eliminate any potential interference that cations (e.g. copper, magnesium, calcium) may have on the reaction (Rice et al., 2017). Briefly, after pH adjustment the pot ale was left to stand for 1 h under continuous shaking (100 rpm) and then re-filtered to 0.2 μm to remove any precipitation. No difference in NH_3–N and NO_3–N concentration was recorded between the pH adjusted (7.0) and non-adjusted (~3.3) pot ale samples (data not shown). TN analysis revealed pot ale contained a high concentration (327–886 mg L^{-1}), which will have come from organic fractions and varied in concentration between the samples. A similar result was reported by Barrena et al. (2017) in which TN concentration varied between 440 and 1100 mg L^{-1} for deproteinated pot ale processed from four independent malt whiskey distilleries (Barrena et al., 2017). The pot ale used in this study also contained high levels of PO_4–P and total phosphorus (TP), with average concentrations of 420.49 ± 50.26 mg L^{-1} and 599.05 ± 66.01 mg L^{-1}, respectively.

3.2. Effect of enrichment with pot ale

3.2.1. Inorganic nutrient removal

Pot ale had a significant effect on the removal of both the NH_3–N and PO_4–P concentrations in PSW inoculated with *C. vulgaris* under static culture conditions. As shown in Fig. 2, a clear depuration of these compounds in the WWPA + *C.v* treatment occurred in all three wastewater batch treatments. In the case of NH_3–N, its concentration declined significantly ($p < 0.05$) and rapidly in the WWPA + *C.v* treatment of PSW batch R1, from an initial 20.9 ± 0.09 mg L^{-1} to 0.09 ± 0.0 mg L^{-1} at day 2 (Fig. 2A). Whereas the NH_3–N concentrations also significantly ($p < 0.05$) decreased in the WWPA + *C.v* treatments for batches R2 and R3, the final concentrations (at day 5) were higher, with initial and final concentrations 47.8 ± 0.09 mg L^{-1} and 17.7 ± 0.9 mg L^{-1} in batch R2 (Figs. 2C) and 35.2 ± 0.03 mg L^{-1} and 4.7 ± 0.2 mg L^{-1} in batch R3 (Fig. 2E). This difference in final NH_3–N concentrations across the different batches (R1, R2, R3) of the WWPA + *C.v* treatment can be attributed to differences in the concentrations of this macronutrient in the collected wastewater, which were higher initially in batches R2 and R3. Tam and Wong (1996) observed a similar response in *C. vulgaris* cultures with varying initial NH_3–N concentrations, in which higher initial concentrations resulted in a lower removal efficiency and consequently higher residual N concentration (Tam and Wong, 1996). We also reported the same response with *C. vulgaris* in our previous study using glucose or glycerol as the organic carbon source for enrichment (Evans et al., 2017).

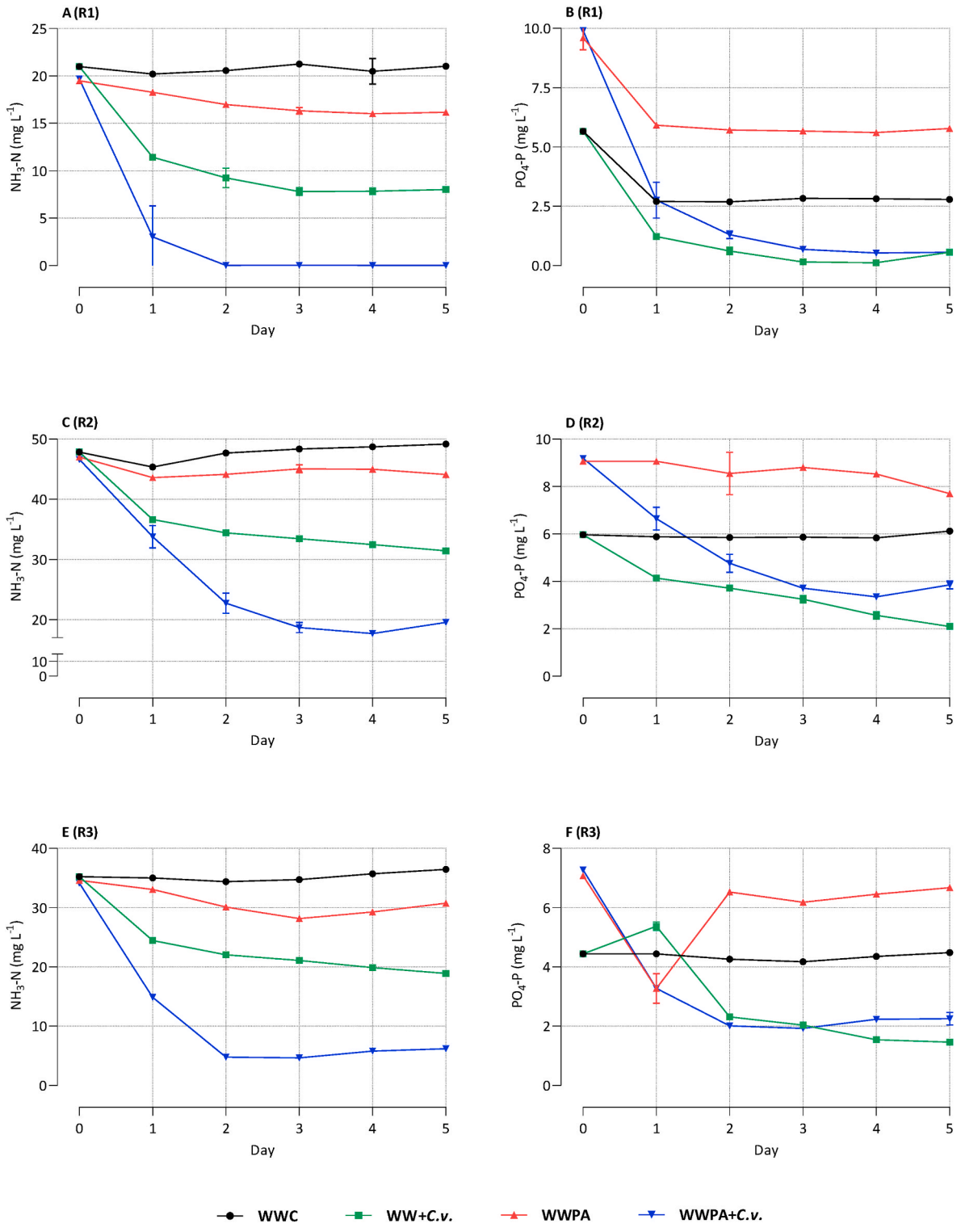

Fig. 2. Changes in PSW concentrations for NH_3–N (A, C, E) and PO_4–P (B, D, F) in mg L^{-1} for PSW batch R1 (A, B), R2 (C, D) and R3 (E, F) treated under the conditions with and without *C. vulgaris*, enriched with or without pot ale. Each data point is the mean ± SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (Wastewater only); Treatment WW + *C.v* (Wastewater with *C. vulgaris*); Treatment WWPA (Wastewater with pot ale); and Treatment WWPA + *C.v* (Wastewater with pot ale and *C. vulgaris*).

The reduced levels of NH_3–N removal in the WWPA + *C.v* treatments of PSW batches R2 and R3 may be a result of the wastewater having become limited in bioavailable carbon for the microalgae to utilise. This inference is based on the trend in COD concentration recorded daily in this experiment. After an initial rapid drop in COD concentrations by day 1 in batches R2 and R3, its rate of decline gradually decreased, with final COD concentrations reaching 154 ± 2.9 mg L^{-1} O_2 and 122 ± 6.6 mg L^{-1} O_2, respectively (Fig. 3 C, E). In these batches, a correlation can be drawn when the concentration of NH_3–N and COD of the WWPA + *C.v* treatments are juxtaposed in respect to their wastewater batch. In the WWPA + *C.v* treatments of PSW batch R2 and R3, the trend in COD concentrations coincided with the decline in NH_3–N concentration, with

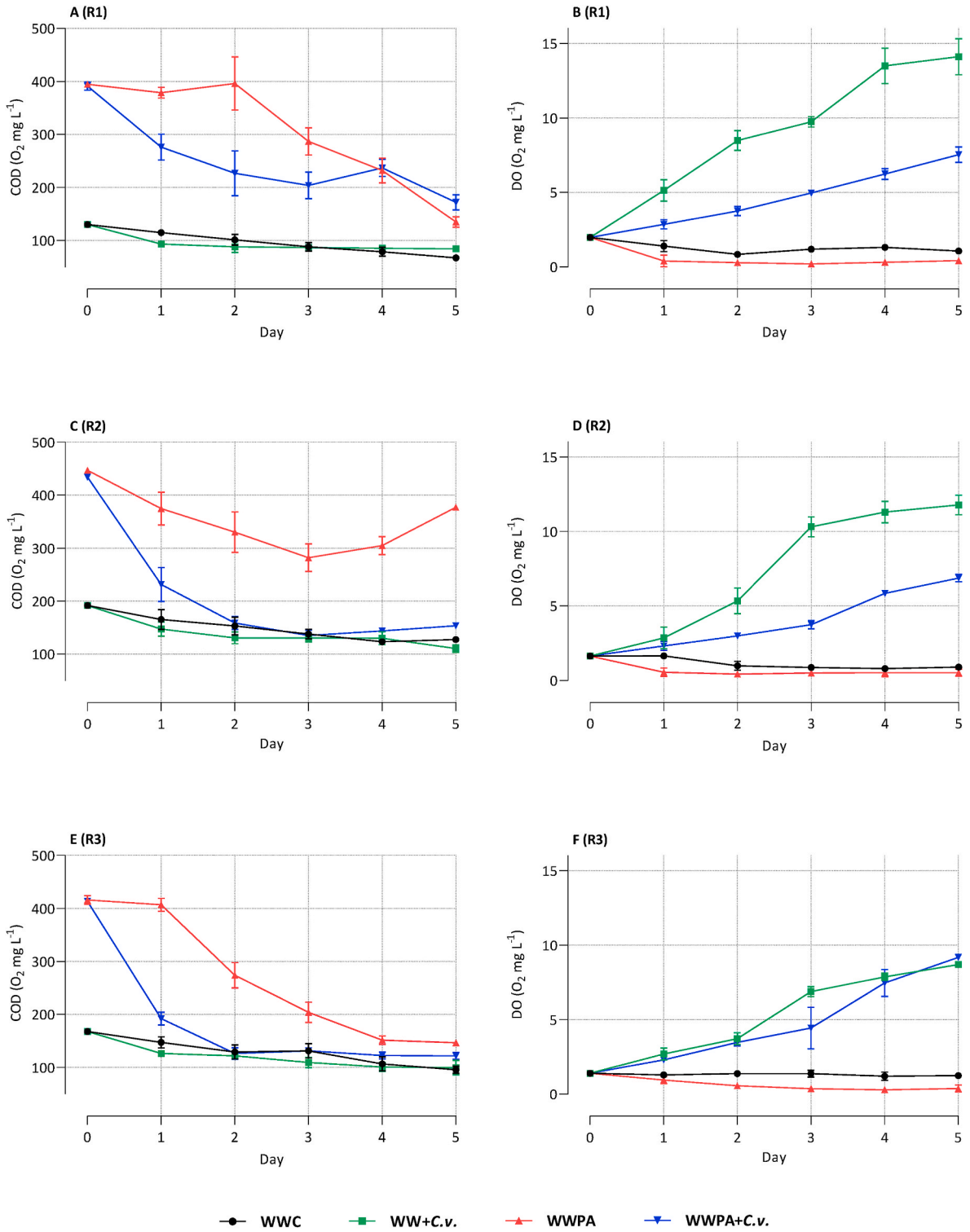

Fig. 3. Changes in COD concentration (A, C, E) and dissolved O_2 concentration (B, D, F) in mg L^{-1} O_2 for PSW batch R1 (A, B), R2 (C, D) and R3 (E, F) treated under the conditions with and without *C. vulgaris*, enriched with or without pot ale. Each data point is the mean ± SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (Wastewater only); Treatment WW + *C.v* (Wastewater with *C. vulgaris*); Treatment WWPA (Wastewater with pot ale); and Treatment WWPA + *C.v* (Wastewater with pot ale and *C. vulgaris*).

the lowest recorded concentration of both parameters occurring at day 3. Thereafter, no substantial change in both the NH_3–N and COD concentrations were recorded for the remaining treatment period, indicating that further carbonaceous material and NH_3–N was not taken up by the microalgal-bacterial co-culture. At this time point (day 3–5) the concentration of COD corresponded in part to the COD concentration recorded in the WW + *C.v* and WWC treatments of the respective PSW batch, from which it can be inferred that the exogenous carbonaceous material in the form of pot ale was almost completely removed. Furthermore, the high concentration of dissolved O_2 in these treatments, compared to in the controls (WWC and WWPA), can be considered as evidence of the near complete depletion of the bioavailable fractions of

carbonaceous material in the PSW as no apparent further degradation occurred (Fig. 2 B, D, F). In the WWPA + *C.v* treatment for batch R1, the trend in COD concentrations was characterised by a slower initial decline until day 3, at which point the concentration slightly increased prior to decreasing again to 172 ± 14.2 mg L^{-1} O_2 at day 5 (Fig. 2 A). The slight increase in COD concentrations at day 4 was likely a result of the accumulation of soluble degradable matter in suspension related to cell death and coincided with a decline in *C. vulgaris* cell concentrations (Figs. 2 A and 4 B).

Similar to the glucose and glycerol enriched treatments in our previous study (Evans et al., 2017), the results from the WWPA + *C.v* treatments suggest a maximum removal capacity of NH_3–N that can be achieved by the microalgal-bacterial co-culture in relation to the quantity of pot ale added. In the WWPA + *C.v* treatments, the total quantity of NH_3–N removed at day 3 was 27.9 mg in PSW batch R2, and 29.5 mg in PSW batch R3. Given that pot ale in both these treatments equated to an approximate 250 mg L^{-1} O_2 COD, it can be inferred that this quantity of carbonaceous material supported the removal of these NH_3–N concentrations from the PSW. In line with this observation, the initial NH_3–N concentration of PSW batch R1 (20.9 mg L^{-1}) was below the typical NH_3–N concentration (90 mg L^{-1}) found in wastewater (Ying et al., 2011), and as such its complete reduction below detection limit in the WWPA + *C.v* treatment was achievable. The C/N ratio in PSW samples ranged between 4 and 6 which is considered lower than the defined C/N ratio (above 8) for a complete and efficient denitrification (Sun et al., 2010). However, the pot ale enriched PSW had a higher C/N ratio ranging between 9 and 20 which can facilitate a more efficient treatment process with the addition of microalgae *C. vulagris* (Zhu et al., 2019). Growth and nutrient removal characteristics of *C. vulgaris* using artificial wastewater have been shown to lead to a complete removal of up to 21.2 mg L^{-1} of NH_4–N, while its removal efficiency dropped 50% at initial concentrations between 41.8 and 92.8 mg L^{-1} NH_4–N (Aslan and Kapdan, 2006). A similar observation was reported by Choi and Lee (2013) using *C. vulgaris* in sterile municipal wastewater amended with ammonium salt. The authors showed a complete reduction in NH_4–N was achieved with an initial concentration of 25.2 mg L^{-1}, and a 50% decline in NH_4–N removal efficiency was recorded in the wastewater at concentrations exceeding 85.5 mg L^{-1}, and which further decreased to less than 30% at concentrations above 105.4 mg L^{-1} (Choi and Lee, 2013). In both these studies, inorganic carbon (as CO_2) was supplied to the medium, either by direct aeration or shaking of the cultures, and as such the cultures were not carbon limited. Collectively, these data suggest that the *C. vulgaris* cannot remediate NH_3–N when its initial concentration is higher than a specific threshold value, but we caution that this effect may be a result of the culture conditions and not directly related to the microalga's physiological or metabolic abilities. Both above studies were conducted in batch cultures with *C. vulgaris* reaching stationary growth, which suggests that N uptake may have been limited because this process is closely related to growth. To ascertain this, it would be interesting to examine if N limitation occurs under continuous cultivation of *C. vulgaris* maintained in a perpetual state of exponential growth.

With regards to phosphorus, the addition of pot ale resulted in a higher initial and consequently final PO_4–P concentration compared to that for the treatments without pot ale enrichment. In the WWPA + *C.v* treatment in PSW batch R1, the concentration of PO_4–P declined rapidly at day 1 to 2.8 ± 0.8 mg L^{-1} after which the rate slowed before reaching a final concentration of 0.5 ± 0.06 mg L^{-1} (Fig. 2B), but which was insignificant compared to the WWPA treatment ($p = 0.243$). Similarly, for the same treatment in PSW batch R3 the highest removal effect was recorded at day 1, declining to 3.3 ± 0.04 mg L^{-1}, with a final PO_4–P concentration of 2.3 ± 0.2 mg L^{-1}, which was found to be significant compared to WWPA (Fig. 2F). Whereas in PSW batch R2 the PO_4–P concentration in this treatment declined at a steadier rate from an initial concentration of 9.1 ± 0.06 to 3.3 ± 0.12 mg L^{-1} at day 4, before slightly increasing to 3.9 ± 0.17 mg L^{-1} at day 5 (Fig. 2D). This decline in PO_4–P concentration was, however, found as insignificant compared to the WWPA treatment. In these treatments the decline in PO_4–P was in part a consequence of microalgal uptake, together with chemical precipitation. Although the precise partitioning of PO_4–P removed by the microalgal-bacterial co-culture and its precipitation was not conducted, the decline in PO_4–P removal rate after the initial days of treatment may have been a response to limited NH_3–N uptake by the microalgae as P assimilation is shown to be dependent on N uptake (Beuckels et al., 2015). In general, final PO_4–P concentration in the WWPA + *C.v* treatments declined to a similar PO_4–P concentration recorded in the WW + *C.v* treatments (Fig. 2 B, D and F).

A divergency in the trend of PO_4–P concentration was observed in the control treatments of batch R3, specifically the WW + *C.v* and WWPA treatments (Fig. 2 F), compared to batches R1 and R2. In the WW + *C.v* treatment of batch R3 a spike in PO_4–P concentration was recorded at day 1, increasing from an initial 4.4 ± 0.07 mg L^{-1} to 5.37 ± 0.14 mg L^{-1} before declining to 2.31 ± 0.07 mg L^{-1} at day 2. Conversely, the PO_4–P concentration in the WWPA treatment declined at day 1 and then doubled at day 2 from 3.27 ± 0.5 mg L^{-1} to 6.52 ± 0.06 mg L^{-1} respectively. Phosphorus in wastewater appears in many forms that can be differentiated into soluble and insoluble fractions composed of reactive phosphorus, acid hydrolysable phosphate and organic phosphorus species, with the concentration of each species varying with respect to the wastewater (Rice et al., 2017). The natural variation in the concentration of these species could explain the spike in the WW + *C.v* treatment at day 1 with higher-than-previous concentrations of organic phosphorus present in the R3 wastewater sample, which is not detectable by the ascorbic acid method used in this study. In this situation the microalgae-bacterial co-culture degraded the organic phosphorus to orthophosphate in the initial 24 h of treatment and utilised it during the remaining period. This scenario would not necessarily be observed in the other treatments of R3 because of variations in their condition. The breakdown of organic phosphorus in the WWC treatment, while not initially, would be limited by the low availability of dissolved oxygen throughout the 5-day treatment period (Fig. 3 F). In comparison, the microalgae-bacterial co-culture in the WWPA + *C.v* treatment was provided with an excess of orthophosphate for the duration of the 5-days that minimised the necessity to expend energy for digesting organic phosphorus. The precise effect that resulted in the drop and rise of PO_4–P in the WWPA treatment is unclear and further analysis that differentiates for all species of phosphorus is needed to better understand the occurrence of such an event.

While it is important to analyse for TP concentration in future studies to ensure the static microalgae treatment process complies with the Urban Wastewater Treatment Directive (Communities, 1991), the high residual concentration of PO_4–P because of pot ale amendment highlights a limitation to the use of this carbon sources in a static microalgae wastewater treatment process. Future work on optimising the static microalgae treatment using pot ale should focus on lowering PO_4–P concentration, and directly TP concentration, to about 2–3 orders of magnitude before the wastewater can be safely discarded into the environment. While microalgae accumulate PO_4–P in the form of polyphosphates, this mechanism increases upon starvation of the cells (Singh et al., 2018). The microalga in the inocula used in these experiments were not starved before being inoculated into the PSW. A potential strategy to improve PO_4–P removal may be to starve the cultures to induce the accumulation of more PO_4–P than the levels demonstrated in these experiments. However, this may have implications upstream of the process that could entail a financial cost because of a further cultivation step required. Alternatively, the P content of the pot ale could be extracted prior to addition in PSW, either through precipitation, adsorption or electrodialysis methods (Parés Viader et al., 2017).

3.2.2. Micro-algae cell growth

Based on the time-course of *C. vulgaris* concentration, the high initial NH_3–N concentration present in PSW batch R2 and R3 combined with

the elevated pH are likely the reasons underlying the slower growth rate and extended lag period exhibited by the microalgae. Based on morphological observations of *C. vulgaris* grown under alkaline conditions, the cell walls of species within this genus (*Chlorella*) may bear greater flexibility to pH-induced effects, and is suggested to prevent rupture of the cells and, therefore, inhibit autospore release (Guckert and Cooksey, 2004). *C. vulgaris* concentrations in the WWPA + *C.v* treatment of PSW batch R3 exhibited a 1-day lag followed by a gradual rate of growth, increasing from 1.2×10^7 ($\pm 1.6 \times 10^6$) cells mL^{-1} at day 1 to a maximum 3.2×10^7 ($\pm 1.9 \times 10^6$) cells mL^{-1} at day 4 (Fig. 4 F). In PSW batch R2, the cell concentration in the WWPA + *C.v* treatment displayed a lower growth rate over the course of the first 4 days of

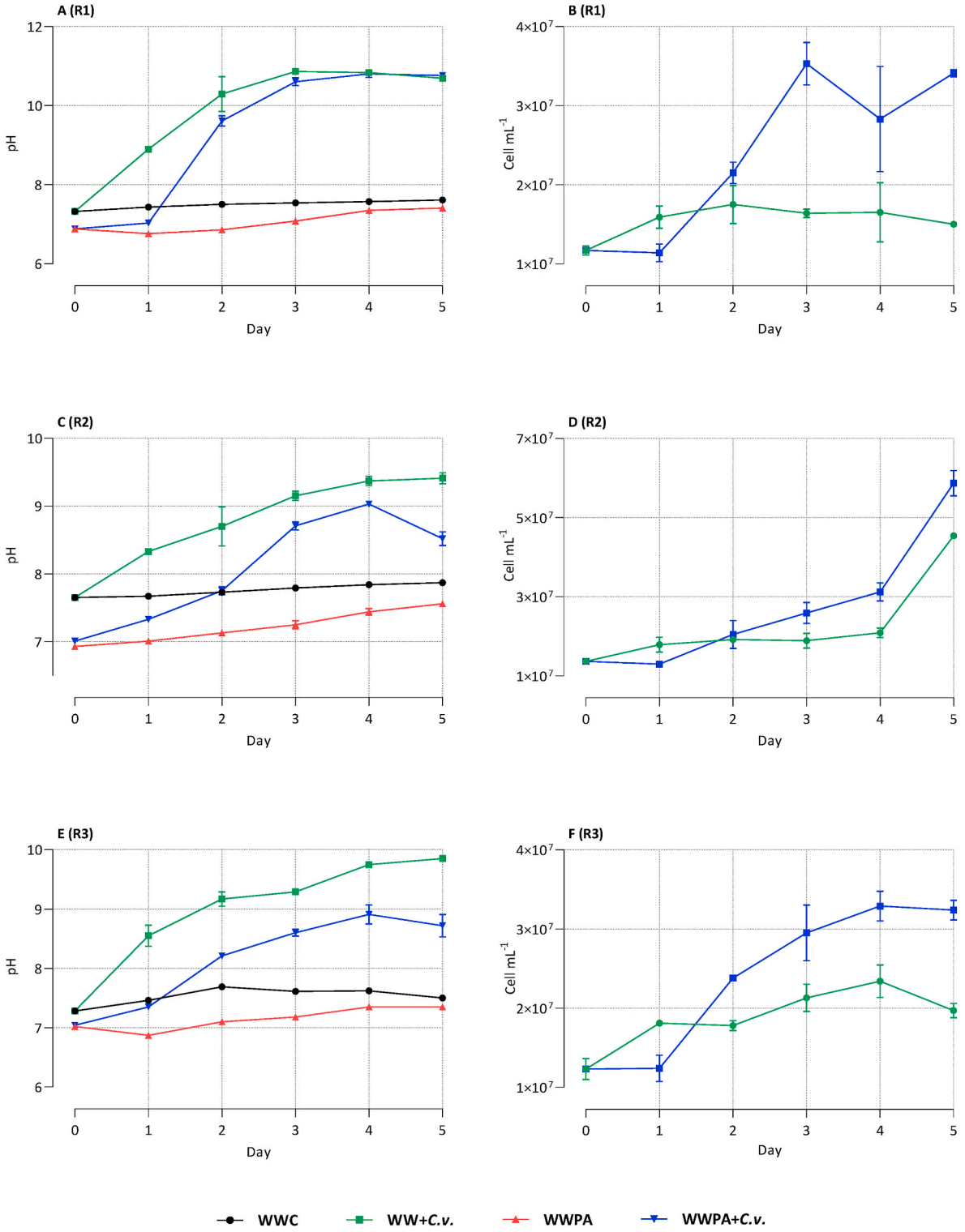

Fig. 4. Change in PSW pH (A, C, E) and *C. vulgaris* concentration (B, D, F) in cell mL^{-1} for PSW batch R1 (A, B), R2 (C, D) and R3 (E, F) treated under the conditions with and without *C. vulgaris*, enriched with or without PA. Each data point is the mean ± SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); Treatment WW + *C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA + *C.v* (wastewater with pot ale and *C. vulgaris*).

treatment, increasing only marginally before substantially increasing at day 5 (Fig. 4 D). Cell concentrations in this treatment were, respectively, 1.3×10^7 ($\pm 5 \times 10^5$) and 3.1×10^7 ($\pm 2 \times 10^6$) cells mL^{-1} at day 1 and 4, and 5.8×10^7 ($\pm 3 \times 10^6$) cells mL^{-1} at day 5. In comparison, cell concentrations in the WWPA + *C.v* treatment of PSW batch R1 (lowest NH_3–N concentrations measured) exhibited a 1-day lag followed by a clear exponential phase, with maximum cell concentrations of 3.5×10^7 ($\pm 2 \times 10^6$) cells mL^{-1} reached by day 3 from initial values of 1.1×10^7 ($\pm 1 \times 10^6$) cells mL^{-1}, followed by a small decline at day 4 before increasing again to an equivalent concentration as recorded on day 3 (Fig. 4 B). At present, we cannot offer a confident explanation to account for the sudden increase in the cell concentration recorded in the WWPA + *C.v* treatment of PSW batch R2 after day 4, yielding the highest cell concentration of all three experimental runs. A similar response was observed in the WW + *C.v* treatment of the same PSW batch, but not for PSW batch R1 or R3, which suggests that the cause may be a result of differential qualities between the collected wastewater used in these batches.

It is interesting to note that despite the discrepancy in *C. vulgaris* concentrations over the 5-day treatment period between the WWPA + *C. v* treatments, the final biomass concentrations in each treatment were similar – 476 ± 25 mg L^{-1}, 410 ± 26 mg L^{-1}, and 426 ± 11 mg L^{-1}, respectively, in PSW batches R1, R2 and R3. The trend in cell growth in the WWPA + *C.v* treatments of PSW batch R2 and R3 bear comparison to microalgae growth under conditions with similar or higher NH_3 concentrations, in which a prolonged acclimation phase or reduced productivity was noted relative to conditions of lower NH_3 concentration (He et al., 2013). For instance, the specific growth rate of *C. vulgaris* cultured on wastewater dropped by a third when the NH_4^+-N concentration was doubled, from 0.92 d^{-1} at 17 mg L^{-1} to 0.33 d^{-1} at 39 mg L^{-1}, displaying a longer acclimation period on a time scale of hours. It must be noted that tolerance to NH_3 is species dependent, which explains the discrepancy in cell growth of *C. vulgaris* observed in the present study when compared to other microalgae wastewater studies with higher NH_3–N concentration in which no effect on microalgae growth and metabolism is observed (Collos and Harrison, 2014). Tolerance of *C. vulgaris* to NH_3–N concentrations of 170 mg L^{-1} or higher have been reported (Aslan and Kapdan, 2006; He et al., 2013).

3.2.3. Dissolved O_2 and pH

The dissolved O_2 concentration increased in the treatments with microalgae, despite the prevailing high free NH_3 formation as a result of pH increase, indicating a prevalence of photosynthetic activity over heterotrophic carbon-oxidation. The WWPA + *C.v* treatments of PSW batches R1, R2 and R3 achieved maximum dissolved O_2 concentrations of 7.5 ± 0.5 mg L^{-1} O_2, 6.8 ± 0.2 mg L^{-1} O_2 and 9.1 ± 0.2 mg L^{-1} O_2, respectively (Fig. 3 B, D and F), and maximum pH values of 10.8 ± 0.09, 9.0 ± 0.02 and 8.9 ± 0.16, respectively (Fig. 4 A, C, E). Previous research has demonstrated that accumulation of free NH_3 in the extracellular environments, which can penetrate internally into algal cells, cause an intracellular pH disturbance, damage PS II and reduce photosynthetic efficiency and O_2 evolution (Drath et al., 2008; Markou et al., 2016). In comparison to the results reported in those studies, it is clear that *C. vulgaris* is tolerant to NH_3 and elevated pH and, hence, demonstrates suitability for wastewater treatment. Despite the presence of O_2 and NH_3–N in the WWPA + *C.v* treatment of PSW batches R2 and R3, the occurrence of nitrification was ruled out based on the absence of no substantial increase in both NO_2–N and NO_3–N concentrations and the elevated pH values present in the wastewater (Supplementary Figure 1). The same observation holds true for the WWPA + *C.v* treatment in PSW batch R1 with the amendment that NH_3–N was limited in the wastewater following its decline at day 2 (Supplementary Figure 1).

3.2.4. Role of pot ale

The concentration of inorganic and organic fractions in pot ale varies between distilleries and their fermentation batches (Traub, 2015; McNerney, 2019). From available studies, the organic carbon fraction in pot ale is found to comprise mostly of organic acids such as acetic acid, propanoic acid and lactic acid following microbial digestion of complex non-fermentative dextrins and solubilized fibre components such as hemicellulose and lignin (White et al., 2020; McNerney, 2019). In microalgae metabolism, these organic acids can be used as carbon sources that are converted to Acetyl-CoA and other precursor compounds through various metabolic pathways which feed into the tricarboxylic acid cycle (TCA) (Falkowski and Raven, 2007; Bashan and Perez-Garcia, 2015). The anabolism of these organic acids has a positive effect on the ability of microalgae to assimilate inorganic nitrogen. It provides them with a greater resource of carbon skeletons in the form of keto acids, taken from the TCA cycle, which are substrates in the anabolism of glutamine and glutamate synthesis. These amino acids are themselves a substrate in the synthesis of other amino acids and form the backbone of inorganic nitrogen assimilation in microalgae. In the present experiment, the greater availability of bioavailable organic carbon supplied by the addition of pot ale can be credited for facilitating the significant rate of NH_3–N removal from the PSW treated by *C. vulgaris*.

It is difficult, however, to precisely determine the individual contribution the microalgae and the heterotrophic (i.e., bacteria, fungi etc.) constituents in the co-culture had in the removal of either the inorganic or organic fractions from the PSW. Characterisation of the wastewater in the microalgae treatments compared with the control treatments (WWC and WWPA) highlight that *C. vulgaris* was a key organism in the consortium responsible for achieving inorganic N and P removal (Fig. 2 A, C, E). In comparison, the WWPA treatments indicate a varied capacity at removing the additional carbonaceous matter provided in the form of pot ale, albeit at a slower rate compared to the WWPA + *C.v* treatments based on COD concentration (Fig. 3 A, C, E). In the WWPA treatments of each PSW batch, a reduction in COD was observed, indicating the ability of the endogenous microbial community in the PSW to digest the carbonaceous matter, including the pot ale (Fig. 3 A, C, E). Bacteria are known to release enzymes extracellularly, as well as express them on their cell membrane, such as glucosidases (e.g. β- and α-amylase), that will have aided in the digestion and removal of the more complex organic compounds present (Burgess and Pletschke, 2008; Mehta and Satyanarayana, 2016). In respect to this, a limitation in the control treatments affecting the endogenous microbial community in the PSW to fully digest carbonaceous matter will have been the anoxic condition that formed. Final dissolved O_2 concentration in the WWPA treatments were 0.42 ± 0.15, 0.53 ± 0.24 and 0.38 ± 0.24 mg L^{-1} O_2 in PSW batch R1, R2 and R3 respectively (Fig. 3 B, D, F). In comparison the concentration of dissolved O_2 increased above 2 mg L^{-1} in the WWPA + *C.v* treatments, achieving required levels for heterotrophic microorganisms to oxidise the organic material, and for autotrophic bacteria to carry out nitrification (Fig. 3 B, D, F) (Metcalf, 2003). Therefore, we posit that the endogenous members of the bacterial community aided in the digestion of the non-fermented dextrins and solubilized fibre components to a form that was more readily available to the microalgae, such as by hydrolysing the polysaccharides into mono- or disaccharides (Bashan and Perez-Garcia, 2015).

Additionally, heterotrophic respiration would have increased the availability of inorganic carbon, although analysis of the individual carbon fractions in the treatments are necessary to more conclusively determine this. Alternative mechanisms of digestion and assimilation of the complex carbonaceous matter by *C. vulgaris* may have been via their extracellular expression of the enzymes necessary for carbohydrate hydrolysis and endocytosis. However, there is a paucity of information detailing the nature or mechanism(s) by which microalgae digest and assimilate more complex carbon substrates in aquatic environments (Bashan and Perez-Garcia, 2015; Y, 2001). Therefore, it cannot be stated with absolute certainty as to whether *C. vulgaris* was able to extracellularly hydrolyse and degrade the non-fermented dextrins and solubilized fibre components of the pot ale. To fully elucidate the mechanism by which the various carbonaceous fractions of pot ale are utilised by the

microalgae, heterotrophic culturing under axenic conditions would need to be performed. A possible investigation to elucidate the mechanism of cellular uptake and verify if the endocytic pathway confers a mechanism for the internalisation of soluble organic carbon, microalgae could be pre-treated with an endocytic inhibitor. In general, the experimental data demonstrates that the microalgae were chiefly responsible for removing the inorganic N and P, and in conjunction with the endogenous microbial community in the PSW, the carbonaceous material.

With respect to the use of brewery or distillery wastewater to grow microalgae, only a few studies have reported on the subject (Choi, 2016; Papadopoulos et al., 2020; Solovchenko et al., 2014; Song et al., 2020; Subramaniyam et al., 2016). Song et al. (2020) reported that microalgae have the potential to simultaneously purify brewery wastewater and fixate carbon dioxide with value-added ingredients co-produced (Song et al., 2020). In addition, Papadopoulos et al. (2020) investigated the bioremediation of brewery wastewater using a cyanobacterial-bacterial consortium and demonstrated a significant reduction of toxicity in algal-treated brewery wastewater (Papadopoulos et al., 2020). Solovchenko et al. (2014) carried out research on a semi-batch operated 50 L microalgae-bacteria treatment process of alcohol distillery wastewater

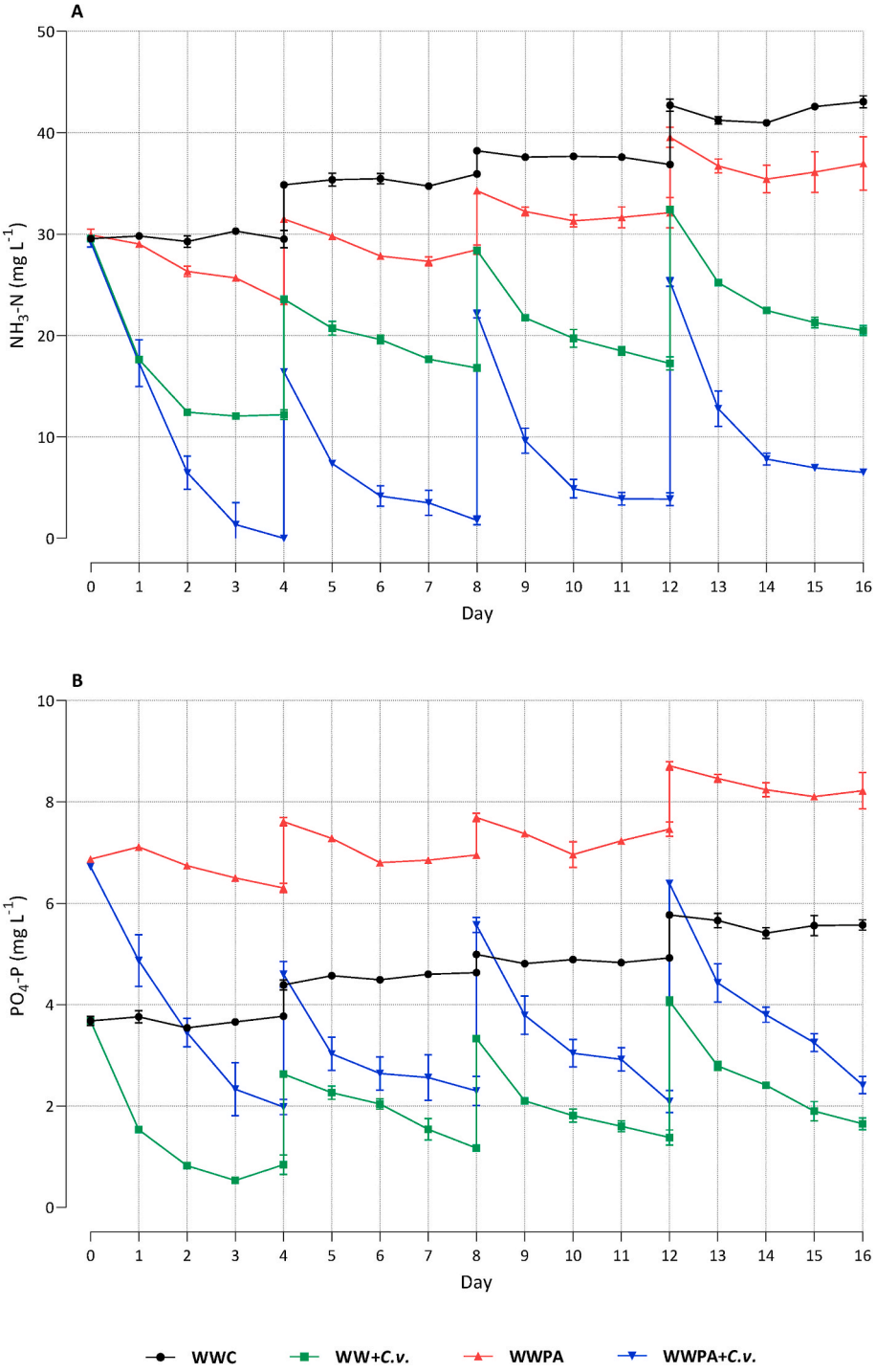

Fig. 5. NH_3–N (A) and PO_4–P (B) concentrations in mg L^{-1} of PSW treated under semi-continuous operation. Each point is a mean ± SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); Treatment WW + *C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA + *C.v* (wastewater with pot ale and *C. vulgaris*).

using the mixotrophic strain *C. sorokiniana* (Solovchenko et al., 2014). In this study, the treatment process was operated for 3 cycles, each run for a period of 4 days, with each cycle achieving significant COD removals, from approximately 20 to 1.5 g L^{-1} O_2. In a preliminary test, alcohol distillery wastewater treated by the endogenous microflora under aerated conditions with atmospheric air achieved no noteworthy COD reduction, demonstrating that the microalgae were vital to reduce the COD. The authors adjusted the pH of the alcohol distillery wastewater to 7.0 to ensure an optimum environment for the microalgae. In the present study, however, adjustment of the pot ale was not necessary, mainly because of the 1:150 dilution factor. Although the addition of pot ale was accompanied by a small decrease in the pH of the wastewater, this did not negatively affect the treatment performance in the WWPA + *C.v* treatment. Other studies have also reported successful results in the treatment of other alcohol-production waste streams using microalgae. For example, Yang et al. (2008) reported a 76% reduction in COD from cassava ethanol fermented wastewater treated by the microalga *C. pyrenoidosa* in both batch and continuous operated mode (Yang et al., 2008). O'Rourke et al. (2016) demonstrated successful mixotrophic cultivation of the microalga *Parachlorella kessleri* on waste residue from fermented wort, with the carbonaceous material composed of residual glucose, maltose and maltodextrin (O'Rourke et al., 2015).

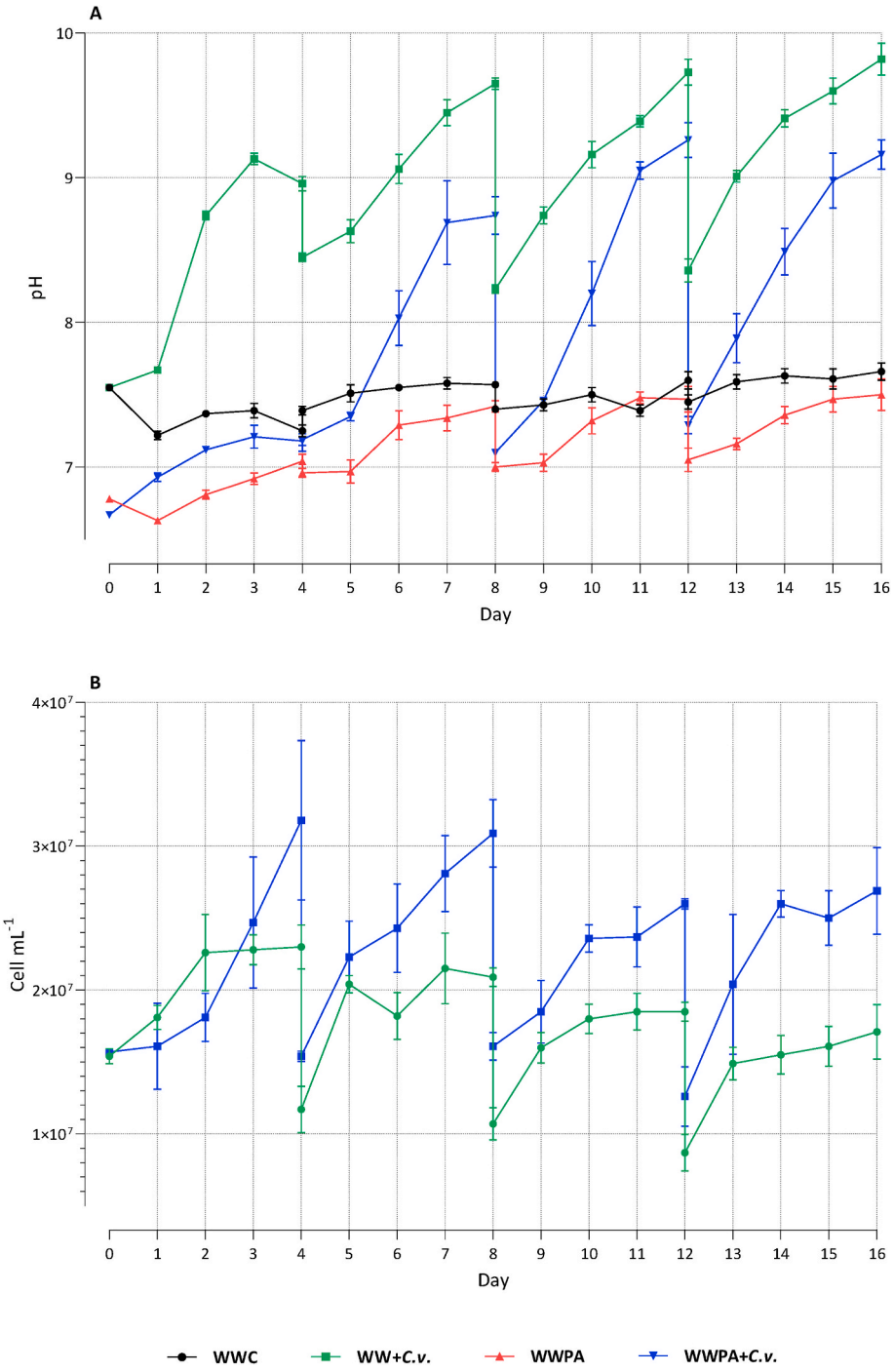

Fig. 6. pH (A) and Cell (B) concentrations in mg L^{-1} of PSW treated under semi-continuous operation. Each point is a mean ± SD, of n = 3 independent replicates. Some error bars are smaller than the symbols. Treatment WWC (wastewater only); Treatment WW + *C.v* (wastewater with *C. vulgaris*); Treatment WWPA (wastewater with pot ale); and Treatment WWPA + *C.v* (wastewater with pot ale and *C. vulgaris*).

3.3. Pot ale enriched PSW treated under semi-continuous mode

Since a semi-continuous (SC) operated process can offer a shorter hydraulic retention time and retainment of an acclimated microalgae-bacterial co-culture, we investigated this as an extension of our batch-wise operated treatments. We found that both the NH_3–N and PO_4–P concentration in the WWPA + *C.v.* SC treatment decreased from initial concentrations of 29.2 ± 0.5 to 0.01 ± 0.01 mg L^{-1} and 6.7 ± 0.07 to 1.9 ± 0.15 mg L^{-1}, respectively, in the first 4 days (cycle 1) (Fig. 5 A, B). This observation is in line with the proposed maximum quantity of NH_3–N removed to the quantity of pot ale added, as discussed in the batch-wise operation (i.e., <30 mg L^{-1} NH_3–N). Thereafter, the efficiency of the treatment declined noticeable by the higher NH_3–N and PO_4–P concentrations recorded at the end of each subsequent cycle (Fig. 5 A, B). Similarly, the COD concentration had an appreciable removal rate of ~75% during cycle 1 and 2 in the WWPA + *C.v.* SC treatment which declined to ~56% in the remaining two cycles (Supplementary Table 1).

Evaluating the treatment efficiency of encapsulated *S. obliquus* in unsterilized urban wastewater, Ruiz-Marin et al. (2010) reported a similar declining effect in a semi-continuous microalgae treatment process. The authors reported a 90% NH_4^+-N removal efficiency within 2 days, which declined to 87% over a further four 2-day cycles before a substantial decline to 10% was recorded in the last cycle (Ruiz-Marin et al., 2010). The decline in removal efficiency in the Ruiz-Marin et al. (2010) study was attributed to the collapse in the microalgae culture, an effect that was also observed in our present study and can be explained by two compounding causes. First, the sharp pH increase recorded in cycles 2 to 4 of the WWPA + *C.v.* SC treatment will have negatively affected the microalgal-bacterial co-culture to grow and assimilate nutrients from the PSW over the course of these cycles (Fig. 6 A). Second, the effect of withdrawing half the culture volume and replacing it with pot ale enriched PSW only diluted the culture by half that resulted in a lower, robust acclimated co-culture concentration that was subjected to the effect of the pH rise. The chronic exposure of the microalgae to dissociated NH_3, because of the high pH, will have affected their health and photosynthetic function, and consequently growth and treatment performance otherwise not noticeable in the 5-day, batch-wise operated treatments. An increase in pH has been shown to negatively affect enzyme activity, nutrient assimilation, and viability and growth of microalgae (Collos and Harrison, 2014; Abeliovich and Azov, 1976; Guckert and Cooksey, 1990). The observed trend of the parameters in the WWPA + *C.v.* treatment supports these assumptions. During cycle 1 of the WWPA + *C.v* SC treatment, the decline in NH_3–N and PO_4–P concentration was accompanied by growth in *C. vulgaris* and biomass concentration over its 4-day duration, with the pH ranging between 6.6 and 7.2 (Fig. 6 A, B). Thereafter, the pH increased to >8.5 in the subsequent cycles and was accompanied with a decline in cell and biomass concentrations. Final cell concentrations in cycles 1, 2, 3, and 4 were, respectively, 3.2×10^7 ($\pm 5.5 \times 10^6$), 3.1×10^7 ($\pm 2.3 \times 10^6$), 2.6×10^7 ($\pm 3.5 \times 10^5$), and 2.6×10^7 ($\pm 3.0 \times 10^6$) cells mL^{-1}, and final biomass concentrations were, respectively, 500 ± 33, 429 ± 45, 424 ± 19 and 321 ± 24 mg L^{-1}. In addition to the maximum cell concentrations reached being lower in each subsequent cycle, the period of arithmetic growth was shorter, lasting only one day (Fig. 6 B). The trend of a higher final NH_3–N and PO_4–P concentrations at the end of each subsequent cycle was also recorded in the WW + *C.v* SC treatment, with a concomitant rise in pH accompanied with a decline in cell concentration. No substantial difference in both the NO_2–N and NO_3–N concentrations were recorded, with the concentrations trending below the detection limit throughout the duration of the experiment for all treatments (Supplementary Figure 2). Overall, controlling the environment that is optimal for the microalgae-bacteria co-culture is essential in developing this approach in further research.

4. Conclusion

This study aimed to evaluate the influence that organic carbon enrichment had on *C. vulgaris* performance to reduce the carbonaceous and inorganic N and P load in PSW under static cultivation conditions and subsequently under a semi-continuous mode. The WWPA + *C.v* treatment demonstrated a significant inorganic N and P removal when compared to the treatments without *C. vulgaris*. With the treatment repeated on three PSW batches, collected and treated separately and sequentially, the final achievable NH_3–N concentration was influenced by its initial concentration and with respect to the quantity of bioavailable carbonaceous matter. Further research on additional wastewater samples with controlled N loads, and adequate pH and dissolved O_2 control measures is needed to draw firmer conclusions with the aim of addressing how to overcome this limitation. Using a readily available organic carbon source in unsterilized PSW presented the possibility of the naturally occurring heterotrophic microorganisms from outcompeting the microalgae. Inclusion of community analysis in any future experiments is recommended to better understand the interaction and influences between the microalgae and other microorganisms under the present experimental design, including the use of non-sterile pot ale. The findings presented here demonstrate that the microalgae were chiefly responsible for removing the inorganic N and P, and in conjunction with the endogenous microbial community in the PSW, the carbonaceous material as well. The use of the deproteinated pot ale as an organic carbon source presents itself as a possible economic solution to treating wastewater by microalgae.

CRediT authorship contribution statement

Laurence Evans: Data curation, Formal analysis, performed experiments, data collection. **Seyedeh Fatemeh Mohsenpour:** Writing – original draft, wrote the article and to which all other authors contributed. **Sebastian Hennige:** designed the experiments. **Nicholas Willoughby:** designed the experiments. **Adebayo Adeloye:** Writing – original draft, wrote the article and to which all other authors contributed, designed the experiments. **Tony Gutierrez:** Designed the experiments, Writing – review & editing, reviewed and revised all versions of the article, and gave final approval for its submission.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work forms part of the Ph.D. research by Laurence Evans, who would like to thank the Water Academy of Heriot-Watt University and a James Watt Scholarship for its financial support. Partial support was also provided through a Natural Environment Research Council grant (NERC, NE/K009028/1) to Sebastian Hennige. The authors are also grateful to Veolia Water Outsourcing Ltd. for their cooperation in providing access to the treatment plant and provision of primary settled wastewater samples. The opinions expressed in this paper are not necessarily those of Veolia Water Outsourcing Ltd. or their operatives. We thank the two anonymous reviewers for their insightful comments which considerably improved the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jclepro.2021.129436.

References

Abeliovich, A., Azov, Y., 1976. Toxicity of ammonia to algae in sewage oxidation ponds. Appl. Environ. Microbiol. (31), 801–806.

Abreu, A.P., et al., 2012. Mixotrophic cultivation of Chlorella vulgaris using industrial dairy waste as organic carbon source. Bioresour. Technol. 187 (118), 61–66.

Aslan, S., Kapdan, I.K., 2006. Batch kinetics of nitrogen and phosphorus removal from synthetic wastewater by algae. Ecol. Eng. 28 (1), 64–70.

Barrena, R., et al., 2017. Batch Anaerobic Digestion of Deproteinated Malt Whisky Pot Ale Using Different Source Inocula. Waste Management, p. 71.

Bashan, Y., Perez-Garcia, O., 2015. Microalgal Heterotrophic and Mixotrophic Culturing for Bio-Refining: from Metabolic Routes to Techno-Economics, pp. 61–131.

Beuckels, A., Smolders, E., Muylaert, K., 2015. Nitrogen availability influences phosphorus removal in microalgae-based wastewater treatment. Water Res. 77, 98–106.

Burgess, J., Pletschke, B., 2008. Hydrolytic Enzymes in Sewage Sludge Treatment: A Mini-Review. Water SA, p. 34.

Cheirsilp, B., Torpee, S., 2012. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: effect of light intensity, glucose concentration and fed-batch cultivation. Bioresour. Technol. 110, 510–516.

Cheng, Y., et al., 2009. Alga-based biodiesel production and optimization using sugar cane as the feedstock. Energy & Fuels 23, 4166–4173.

Choi, H.-J., 2016. Parametric study of brewery wastewater effluent treatment using Chlorella vulgaris microalgae. Environmental Engineering Research 21 (4), 401–408.

Choi, H., Lee, S.-M., 2013. Performance of Chlorella Vulgaris for the Removal of Ammonia-Nitrogen from Wastewater, vol. 18. Environmental Engineering Research.

Collos, Y., Harrison, P.J., 2014. Acclimation and toxicity of high ammonium concentrations to unicellular algae. Mar. Pollut. Bull. 80 (1–2), 8–23.

Communities, C.o.t.E., 1991. EU, Urban Wastewater Directive 91/271/EEC. Official Journal of the European Communities.

Drath, M., et al., 2008. Ammonia triggers photodamage of photosystem II in the cyanobacterium Synechocystis sp. strain PCC 6803. Plant Physiol 147 (1), 206–215.

Evans, L., et al., 2017. Effect of organic carbon enrichment on the treatment efficiency of primary settled wastewater by Chlorella vulgaris. Algal Research 24, 368–377.

Falkowski, P.G., Raven, J.A., 2007. Aquatic Photosynthesis, second ed. Princeton University Press, New Jersey, USA.

Gélinas, M., 2015. Residual corn crop hydrolysate and silage juice as alternative carbon sources in microalgae production. Algal research 12, 33–42, 2015 v.12.

Guckert, J.B., Cooksey, K.E., 1990. Triglyceride accumulation and fatty acid profile chang-es in Chlorella (Chlorophyta) during high pH-induced cell cycle inhibition. J. Phycol. (26), 72–79.

Guckert, J., Cooksey, K., 2004. Triglyceride accumulation and fatty acid profile changes in Chlorella (Chlorophyta) during high pH-induced cell inhibition. J. Phycol. 26, 72–79.

He, P.J., et al., 2013. Cultivation of Chlorella vulgaris on wastewater containing high levels of ammonia for biodiesel production. Bioresour. Technol. 129, 177–181.

Liang, Y., Sarkany, N., Cui, Y., 2009. Biomass and lipid productivities of Chlorella vulgaris under autotrophic, heterotrophic and mixotrophic growth conditions. Biotechnol. Lett. 31, 1043–1049.

Lutzu, G.A., Zhang, W., Liu, T., 2015. Feasibility of using brewery wastewater for biodiesel production and nutrient removal by Scenedesmus dimorphus. Environ. Technol. 37, 1–54.

Markou, G., Depraetere, O., Muylaert, K., 2016. Effect of ammonia on the photosynthetic activity of Arthrospira and Chlorella: a study on chlorophyll fluorescence and electron transport. Algal Research 16, 449–457.

McNerney, C.C., 2019. Exploring the Potential Resource Recovery from Pot Ale Utilising Microalgae and Novel LED Photobioreactors. Robert Gordon University.

Mehta, D., Satyanarayana, T., 2016. Bacterial and archaeal α-amylases: diversity and amelioration of the desirable characteristics for industrial applications. Front. Microbiol. 7, 1129, 1129.

E. H Metcalf, E., 2003. Wastewater Engineering: Treatment and Reuse, fourth ed. McGraw-Hill Higher Education, Columbus, USA.

Mohana, S., Acharya, B.K., Madamwar, D., 2009. Distillery spent wash: treatment technologies and potential applications. J. Hazard Mater. 163 (1), 12–25.

Mohsenpour, S.F., et al., 2020. Integrating micro-algae into wastewater treatment: a review. Sci. Total Environ. 752, 142168.

O'Rourke, R., Gaffney, M., Murphy, R., 2015. The Effects of Parachlorella Kessleri Cultivation on Brewery Wastewater, vol. 73. Water Science and Technology.

Pant, D., Adholeya, A., 2007. Biological approaches for treatment of distillery wastewater: a review. Bioresour. Technol. 98 (12), 2321–2334.

Papadopoulos, K.P., et al., 2020. Brewery wastewater treatment using cyanobacterial-bacterial settleable aggregates. Algal Research 49, 101957.

Parés Viader, R., et al., 2017. Sequential electrodialytic recovery of phosphorus from low-temperature gasification ashes of chemically precipitated sewage sludge. Waste Manag. 60, 211–218.

Perez-Garcia, O., et al., 2011. Heterotrophic cultures of microalgae: metabolism and potential products. Water Resour. (45), 11–36.

Rice, E.W., Baird, R.B., Eaton, A.D., 2017. Standard Methods for the Examination of Water and Wastewater, 23rd Edition. American Public Health Association, American Water Works Association, Water Environment Federation.

Ruiz-Marin, A., Mendoza-Espinosa, L., Stephenson, T., 2010. Growth and nutrient removal in free and immobilized green algae in batch and semi-continuous cultures treating re-al wastewater. Bioresour. Technol. (101), 58–64.

Sforza, E., et al., 2012. Excess CO2 supply inhibits mixotrophic growth of Chlorella protothecoides and Nannochloropsis salina. Bioresour. Technol. (104), 523–529.

Singh, D., Nedbal, L., Ebenhöh, O., 2018. Modelling phosphorus uptake in microalgae. Biochem. Soc. Trans. 46 (2), 483–490.

Solovchenko, A., et al., 2014. Phycoremediation of alcohol distillery wastewater with a novel Chlorella sorokiniana strain cultivated in a photobioreactor monitored on-line via chlorophyll fluorescence. Algal Research 6, 234–241.

Song, C., et al., 2020. Combination of brewery wastewater purification and CO2 fixation with potential value-added ingredients production via different microalgae strains cultivation. J. Clean. Prod. 268, 122332.

Subramaniyam, V., et al., 2016. Cultivation of Chlorella on brewery wastewater and nano-particle biosynthesis by its biomass. Bioresour. Technol. 211, 698–703.

Sun, S.-P., et al., 2010. Effective biological nitrogen removal treatment processes for domestic wastewaters with low C/N ratios: a review. Environmental Engineering Science - Environ Eng Sci 27, 111–126.

Tam, N.F.Y., Wong, Y.S., 1996. Effect of ammonia concentrations on growth of Chlorella vulgaris and nitrogen removal from media. Bioresour. Technol. 57 (1), 45–50.

Traub, J.E., 2015. Protein Recovery from Whisky by - Products : A Study of Using Ion Exchange Chromatography for the Recovery of Proteins from Pot Ale. Heriot-Watt University.

Wei, A., et al., 2009. Effects of cassava starch hydrolysate on cell growth and lipid accumulation of the heterotrophic microalgae Chlorella protothecoides. J. Ind. Microbiol. Biotechnol. 36 (11), 1383–1389.

Westwood, 2007. The Determination of Chemical Oxygen Demand in Waters and Effluents. E. Agency.

White, J.S., et al., 2016. Chapter 13 - recovery and applications of proteins from distillery by-products. In: Singh Dhillon, G. (Ed.), Protein Byproducts. Academic Press, pp. 235–253.

White, J.S., et al., 2020. Characterization of pot ale from a scottish malt whisky distillery and potential applications. ACS Omega 5 (12), 6429–6440.

Y, L., 2001. Microalgal mass culture systems and methods : their limitation and potential water flush. Applied Phycology (13), 307–315.

Yang, C.-f., Ding, Z.-y., Zhang, K.-c., 2008. Growth of Chlorella pyrenoidosa in wastewater from cassava ethanol fermentation. World J. Microbiol. Biotechnol. 24, 2919–2925.

Ying, L., Ai-jun, G., Jing, Z., 2011. Study of treating high ammonia-N domestic wastewater with CASS process. Procedia Environmental Sciences 11, 858–863.

Zhan, J., Rong, J., Wang, Q., 2017. Mixotrophic cultivation, a preferable microalgae cultivation mode for biomass/bioenergy production, and bioremediation, advances and prospect. Int. J. Hydrogen Energy 42.

Zhu, S., et al., 2019. Treatment of low C/N ratio wastewater and biomass production using co-culture of Chlorella vulgaris and activated sludge in a batch photobioreactor. Bioresour. Technol. 274, 313–320.