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Influence of season, inoculum density and ammonia concentration on biomass abundance and ammonia utilization of a microalgal consortium for tertiary remediation of tannery wastewater and valorization of biomass

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ABSTRACT

Phyco-remediation has been advocated as a tertiary step for simultaneous remediation of tannery wastewater and valorization of microalgal biomass. This study was based on the hypothesis that climatic factors could be important functional determinants for system performance in open phyco-remediation systems subject to seasonal variations. In this study, ammonia utilization and biomass abundance obtained by a microalgal consortium (*Tetradesmus obliquus* CCAP 276/1A, *Tetradesmus obliquus* CPUT-L1, *Tetradesmus dimorphus* CPUT-L2, *Neochloris* sp. CPUT-W1) in treated tannery wastewater were measured under simulated winter, autumn/spring and summer conditions (temperature: 8.0 °C, 17.5 °C, 27.0 °C, respectively; photoperiod: 10:14 h, 12:12 h, 14:10 h light:dark, respectively) with varied initial ammonia (7.4 mg/L to 45.6 mg/L) and inoculum size (6 % vol./vol to 34 % vol./vol.) in 500 mL flasks (350 mL working volume) in shaking incubators at 160 rpm. Season had highly significant effects ($p < 0.0001$) on both responses in an increasing continuum from winter to summer (biomass: 0.39 ± 0.06 g/L to 0.72 ± 0.07 g/L; ammonia utilization 1.7 ± 0.6 mg/day to 4.1 ± 1/6 mg/day). The inoculum size did not have significant effects on either response ($p > 0.5$), while the initial ammonia concentration had a highly significant effect ($p < 0.0001$) on the ammonia utilization rate, but not on biomass abundance. Despite the halo-tolerant nature of the microalgal strains, sodium removal efficiency was <1 %, indicating that it is unlikely that phyco-remediation is feasible for removal of sodium in highly saline wastewaters. Overall, this study demonstrated the importance of studying microalgal growth and remediation under climatic conditions likely to be expected in the field with a view to pilot scale implementation.

1. Introduction

Approximately 200 kg of leather, 500 kg of wet sludge, and 50–15,000 m^3 of tannery wastewater (TWW) is generated from processing each ton of raw hides/skins in tanneries [1]. Tannery wastewater generated from conventional beamhouse and tanning operations requires extensive remediation before discharge because it is highly saline and contains toxic metals and high concentrations of organics, sulfur (S) species and ammonia/ammonium (NH_3/NH_4) [2]. Discharge of improperly treated TWW can have an adverse effect on the environment and human health as toxic metals, including chromium (Cr), zinc (Zn), lead (Pb) and cadmium (Cd) can accumulate in soils and enter aquatic environments [3]. Conventional treatment strategies include primary, secondary and tertiary processes and are site specific [4]. The conventional activated sludge process is favored for secondary remediation, but the process is energy intensive, and tanneries struggle to comply with local discharge standards unless expensive ancillary physicochemical processes are applied [4]. Anaerobic digestion is gaining traction as techniques for reducing process inhibition by toxins in TWW are being identified [4]. Some microalgal species have shown promise for tertiary phyco-remediation of TWW because they produce enzymes that enable them to thrive in extreme metal- and salt-containing environments [5]. Microalgae exhibit diverse trophic modes, including autotrophy, heterotrophy, and mixotrophy, enabling adaptability to various environmental conditions [6,7]. Studies have assessed the effects of biotic and abiotic factors on microalgal growth

Abbreviations: CODt, total chemical oxygen demand; CODs, soluble chemical oxygen demand; DCW, dry cell weight; GLM, generalized linear model; IS, inoculum size; RCM, reduced cubic model; $[NH_3]_{initial}$, initial ammonia concentration; TWW, tannery wastewater; TTWW, treated tannery wastewater.

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and pollutant removal from TWW [8,9]. Despite the adaptability of microalgae, laboratory studies suggest that secondary phyco-remediation of TWW is not feasible because robust microalgal growth has only been achieved after diluting TWW [10,11], mixing TWW with sewage [12], or after pre-treatment of TWW via ozonation [13,14]. However, tertiary phyco-remediation and biomass valorisation in integrated TWW-biorefineries may be a viable option, as microalgae have been successfully grown in TWW following secondary treatment [15,16]. Growing microalgal biomass in TTWW as a culture medium promotes a circular bioeconomy, utilizing waste as a resource while improving the quality of the effluent for discharge [17].

Ammonia, especially in the unionized form, is highly toxic to aquatic organisms [18]. The relative contribution of NH_3 to total ammonia nitrogen (TAN) increases 10-fold for each unit increase in pH [18], so that alkaline TWW from beamhouse operations can be highly toxic [2,19]. In the context of TWW biorefineries, pre-treatment for removal of sulfur (S) species in integrated hybrid linear flow channel reactors followed by anaerobic digestion (AD) of TWW has been proposed for simultaneous TWW remediation coupled with recovery of bio-sulfur and energy [20,21]. However, hydrolytic reactions in one-stage AD further elevates the NH_3/NH_4 levels in the TWW [20–22]. In addition, secondary treatment does not remove sodium (Na) from TWW, and soil structures are negatively impacted over time when saline effluent is discharged to land environments [23]. It is therefore important to reduce the amount of NH_3/NH_4 and Na in treated tannery wastewater (TTWW) before discharge.

Nitrogen is utilized by microalgae for the synthesis of pigments, proteins, and nucleic acids, with N constituting 1 % to 14 % of their dry weight [24,25]. Ammonia is the preferred source of N for most microalgal species [24,25] which is why phyco-remediation has been ubiquitously applied for bioremediation of effluents that contain high concentrations of NH_3, such as piggery wastewater [26] and landfill leachates [27]. Halo-tolerant microalgae can utilize NH_3 and absorb or adsorb Na, as demonstrated by strains of *Chlorococcum humicola* and *Chlorella vulgaris* [28], and *Scenedesmus (*now *Tetradesmus) obliquus* [29], among others.

For this study, it was hypothesized that a microalgal consortium could be used for tertiary removal of NH_3/NH_4^+ and Na from TTWW with possible generation of utilizable microalgal biomass. The study was based on the premise that it is not cost-effective for tanneries to run sophisticated microalgal bioreactors and that TWW-microalgal biorefineries would need to utilize more cost-effective non-temperature controlled open systems such as raceway ponds. Such systems are subject to climatic variability, which can influence the growth, metabolism, and succession of strains within microalgal consortia. This fundamental study sought to provide insight into how variability in climatic conditions may affect removal of NH_3 and the character and volume of the biomass that is generated as a prelude to piloting the technology. For this study, four microalgal strains were selected from over 20 promising strains for their ability to thrive in TTWW without nutrient addition [30]. In theory, consortia are more resilient than individual strains, because different strains within the consortium may functionally dominate as environmental conditions change. A central composite design (CCD) experiment was conducted to assess the effects of seasonality, initial NH_3 concentration ($[NH_3]_{initial}$) and inoculum size (IS) on: (i) biomass quality and quantity, and (ii) the NH_3 utilization rates of the strains applied as a consortium. The biomass generated during each simulated season was comprehensively characterized and the results were analysed in terms of potential downstream applications. The intention of the study was not to optimize the growth conditions, but to ascertain how processes may differ under different simulated seasonal conditions based on real temperatures and photoperiods. A consortium was applied because it has been established that monocultures are less resilient to system perturbations and achieve lower pollutant removal rates [31]. In addition, it is impossible to maintain monocultures in open systems [31].

2. Materials and methods

2.1. Microalgal consortium

The microalgal consortium consisted of one commercial microalgal strain (*Tetradesmus obliquus* CCAP 276/1A (formerly *Scenedesmus basiliensis* CCAP 276/1A) and three in-house strains. The in-house strains were previously identified using molecular methods and the sequences are available in the National Centre for Biotechnology Information (NCBI) database under Project PRJNA1172546: *Tetradesmus obliquus* CPUT-L1 (NCBI accession number SAMN44272498), *Tetradesmus dimorphus* CPUT-L2 (NCBI accession number SAMN44272499) and *Neochloris* sp. CPUT-W1 (NCBI accession number SAMN44272500) [30]. The four strains were selected from a range of halotolerant strains for their ability to grow robustly in TTWW without nutrient addition [30].

2.2. Experimental design and procedures

The consortium was first used in a flask-based batch experiment based on CCD to determine the effects of seasonality, $[NH_3]_{initial}$ and IS on the NH_3 utilization rate, biomass quantity and TWW remediation potential of the consortium. The combinations of factors/variables resulting in the highest and lowest amounts of biomass for each season were then increased from 350 mL to 600 mL, mainly to generate sufficient biomass for characterization purposes. The remediation potential under each set of conditions was compared and the biomass harvested from the increased volume was comprehensively characterized (Section 2.3).

The inoculum was prepared from cultures of the four individual microalgal strains grown in TTWW for 30 days after acclimation to increasing concentrations of raw TWW diluted with bold basal medium (BBM) as described by [30]. The biomass of each strain was determined by measuring the optical density (OD) at 680 nm and converting into g/L using individualized standard graphs as previously described [30]. To ensure equal densities of each strain in the inoculum, the three *Tetradesmus* strains were each diluted to the same concentration (0.73 g/L) as the *Neochloris* sp. CPUT-W1 strain (viz. the strain with the lowest concentration). All cultures were then pooled in equal volumes. This was the highest density that could be obtained while ensuring that all strains were present in the same concentrations. The $[NH_3]_{initial}$ range was based on historical data obtained from the tannery used in this study.

The experiments were conducted using sterilized TTWW as the growth medium under different simulated climatic (Table 1) and physicochemical conditions (Table S1). The CCD experimental runs were conducted in 500 mL Erlenmeyer flasks (350 mL working volumes) for 13.5 days, while for the biomass harvesting, the consortia were grown in 1 L flasks (600 mL working volumes) in duplicate for 19.5 days. All flasks were incubated in shaking incubators at 160 rpm (rpm). Light was supplied by Sylvania® (Newhaven, United Kingdom) grow bulbs that emit 31 umol/m^2/s photosynthetic photon flux (PPF) of light in the deep red, far red, blue, and white ranges of the visible spectrum. A separate shaking incubator was used for each season. Temporal variations in the temperatures and photoperiods were not accounted for in the experimental design.

Table 1
Temperatures and photoperiods applied for different simulated seasons.

Simulated season[a]	Temperature	Photoperiod	
		Light	Dark
Summer	27 °C	14 h	10 h
Spring/autumn	17.5 °C	12 h	12 h
Winter	8 °C	10 h	14 h

[a] Based on average climatic data for Cape Town 2013–2023 obtained from the from South African Weather Services (SAWS) (personal communication).

For the CCD experiment, the software Design Expert® version 13 (Stat-Ease, Inc., Minneapolis, USA) was used to generate 36 experimental runs (Table S1). The design included replicates for summer (runs 8,16,18,21), autumn/spring (runs 5, 9, 17, 27), and winter (runs 4, 23, 25, 33). The two numerical variables, $[NH_3]_{initial}$ (15–40 mg/L) and IS (10–30 % vol./vol.) were set to two levels (plus and minus 1 factorial point), along with a central point. The categorical factor (season) was tested at three levels corresponding to winter, autumn/spring, and summer. The responses were evaluated based on two parameters: NH_3 utilization rate (mg/day) and biomass abundance (g/L).

2.3. Physicochemical analyses

2.3.1. Sampling schedule

Samples of TTWW were taken at the start of each experiment. For the CCD experiment, 2 mL aliquots of flask contents were extracted aseptically every 3 days for measurements of pH, total alkalinity (T.alk) and NH_3. The remaining parameters for the CCD experiment were measured in samples taken at the end of the 13.5-day experimental period: total and soluble chemical oxygen demand (CODt, CODs), sulfate (SO_4^{2-}), total nitrogen (TN), and volatile organic acids (VOA). In addition, the CODt, CODs and NH_3 were measured in the contents from the 1 L flasks at the end of the 19.5-day experimental period.

The pH, CODt, and TN were performed on whole samples, while the other TWW analyses were conducted on filtered samples (soluble fraction). The centrifugate (biomass) was dried in an oven at 65 °C overnight and crushed to a fine powder using a pestle and mortar for characterization.

2.3.2. Analytical procedures

The T.alk, CODt, CODs, SO_4^{2-}, TN, VOA and NH_3 analyses were conducted on the TTWW (influent) and flask contents using a Merck Spectroquant Pharo instrument (Merck, Darmstadt, Germany) together with standards and controls as per the manufacturers' instructions as previously described [32]. The pH was measured using a Eutech pH 700 instrument and pH probe (Eutech instruments, Singapore). Major and minor cations (Table 4) were quantified at the Central Analytical Facility (CAF) at Stellenbosch University (Stellenbosch, South Africa) in the filtered samples and extracts of the dried biomass using a Thermo ICap 6200 ICP-AES instrument for trace analyses, and an Agilent (Santa Clara, USA) 7900 ICP-MS instrument for ultra-trace analyses together with standards and controls as specified by the manufacturer. The carbon, hydrogen, nitrogen, (CHN) content (%wt./wt.) of the biomass was quantified at CAF using an Elementar (Hamburg, Germany) Vario EL cube Elemental analyser according to the manufacturers' instructions as previously described [30].

The protein content of the biomass was determined by multiplying the N content by a factor of 5.8 [33]. The carbohydrate content was determined using the Anthrone spectroscopic method as described by Chen & Vaidyanathan [34]. The fatty acids were qualitatively and quantitatively evaluated as previously described [30] using an Agilent 7890 A Gas Chromatography–Flame Ionisation Detector (GC-FID) System with a HP88 (100 m × 250 μm, 0.250 μm) column. Nitrogen was used as the carrier gas at a flow rate of 1.0 mL/min and an injection volume of 1 μL (split 100:1). The oven temperature program was set to hold at 100 °C for 5 min, to increase at 3 °C/min until 250 °C was reached, and then to hold for a further 20 min [30].

2.3.3. Biomass quantification

The amount of biomass was determined gravimetrically at the end of the experimental period by transferring 100 mL of well-mixed flask contents to a sterile pre-weighed container, drying at 65 °C overnight and deducting the weight of the container from the weight of the container with biomass. The biomass was also semi-quantified spectroscopically using chlorophyll-a as a proxy by measuring the optical density (OD) at 680 nm. In addition, cell counts were performed on sample aliquots using a Neubauer chamber according to the method described by [35].

2.4. Statistical analyses and models

A reduced cubic model (RCM) based on CCD was used to explore relationships between factors and responses in the 36 experimental runs using Design Expert® version 13 software. Notably, no blocks were incorporated in the design. Non-significant data was eliminated from the RCM before performing analysis of variance (ANOVA) on the model outputs. Normality testing of the variables was conducted using the Shapiro-Wilk test and model effects of a generalized linear model (GLM) were determined using SPSS (IBM, New York, USA) version 29 software. Correlations between physicochemical parameters were determined using Microsoft Excel (*t*-test paired samples for equal means) and regression analyses.

3. Results and discussion

3.1. Microalgal growth and effects of season, initial ammonia concentration and inoculum density on biomass abundance

Provided adequate material is available, gravimetric measurement of the dry cell weight (DCW) is the gold standard for monitoring growth of microalgae [35,36]. Two other simple methods that are commonly utilized as proxies for determining the concentrations of microalgal biomass are the spectroscopic estimation of chlorophyll-a, and microalgal cell counts [35,36]. Although widely used, both are subject to inconsistencies. Cell counts do not consider the size of the cells, which may theoretically lead to anomalous results in terms of biomass. Although good linear correlations ($R^2 > 0.99$) were previously obtained between DCW and chlorophyll-a (OD at 690 nm) for the individual microalgal strains used in this study, the slopes were inconsistent from strain to strain, strongly suggesting that the method may be inaccurate for monitoring growth of microalgal consortia [30]. However, the relatively small working volumes used in the CCD experiments (350 mL) could not cater for regular extraction of sufficient amounts of flask contents for accurate temporal DCW measurements. Despite the perceived limitations, cell counts and chlorophyll-a measurements were assessed as proxy methods for determining the growth profiles and biomass productivity rates in this study.

Moderate overall correlations were found between both proxy methods and DCW, but intra-seasonal discrimination was poor with generally low correlations (Table 2, Fig. 1). The highest Pearsons' correlation co-efficient ($R = 0.870$) and regression analysis value ($R^2 = 0.757$) were found between chlorophyl- a and DCW. The correlation was lowest in the winter simulated runs. This may be explained by the lower photoperiod applied in winter, as light is required for synthesis of chlorophyll. In winter, more heterotrophic growth and less autotrophic (photosynthetic) growth would be expected than in spring-autumn or summer.

In terms of cell counts: (i) the sizes of the different strains of microalgal cells differed, (ii) many of the microalgal cells formed coenobia, making counting difficult, and (iii) the microalgal cells exhibited seasonal size differences, with most tending to be larger in the summer-simulated than in the winter-simulated conditions (not shown). It was concluded that neither of the proxy methods were sufficiently accurate for determining temporal changes in the biomass of the microalgal consortium, which is probably also relevant for other studies employing microalgal consortia. For these reasons, the DCW (response: biomass) at the end of the study was used to assess growth of the consortium under the varied conditions. Kinetics were not assessed because the growth profiles could not be determined, and it was expected that the phases of growth (lag, log, stationary) would differ from culture to culture, skewing the data.

The amount of biomass measured at the end of the study ranged from

Table 2
Pearsons' correlations (R-values) between biomass proxy methods and dry cell weight.

Overall		Winter		Spring/autumn		Summer	
Chlorophyll	Count	Chlorophyll	Count	Chlorophyll	Count	Chlorophyll	Count
0.870*	0.691*	0.108*	0.179*	0.447*	0.070*	0.605*	0.147*

Paired 2-samples for means, chlorophyll *a* (OD) at 680; count = microalgal cell count (cells/mL) *p < 0.001 for all, T-stat>T-crit for all.

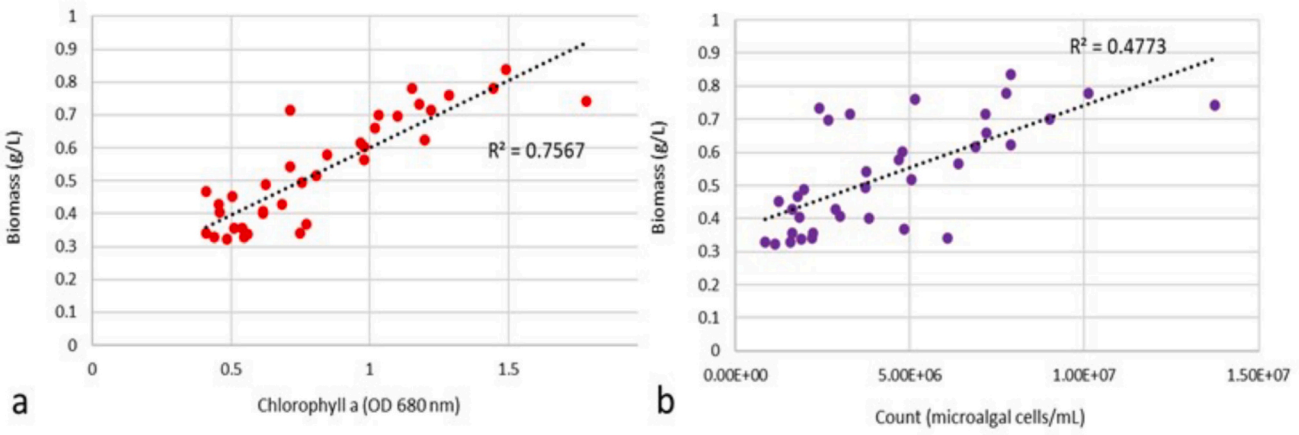

Fig. 1. Scatter plots of dry cell weight plotted against chlorophyll-a concentration (a) and microalgal cell counts (b).

0.38 ± 0.06 to 0.50 ± 0.11 to 0.72 mg/L in the winter- spring-autumn and summer-simulated runs, respectively. Results compared well with those obtained in wastewater studies ($n = 10$) with *T. obliquus*, where the DCW ranged from 0.005 g/L in municipal wastewater [37] to 3.8 g/L in poultry wastewater [38]. Results also compare favorably with similar strains grown in artificial media: (i) 0.68 g/L and 1.0 g/L of *T. obliquus* NEIST BT-A1 and *T. dimorphus* NEIST BT1 grown in flasks in BG 11 medium [39], (ii) 0.56 to 20 g/L *T. obliquus* in photobioreactor studies ($n = 10$) reviewed by Oliveira et al. (2021) [38], the highest being in a laboratory cascade system fed with Laboratory of Algae Culture (LCA) medium [40], (ii) 0.40 g/L and 0.54 g/L of *Neochloris pseudostigmata* UTEX 1249 and *Neochloris conjuncta* CCAP 254/1, respectively in 40 % diluted synthetic centrate in Bristol medium [41], and 0.22 to 0.89 g/L *Neochloris oleoabundans* UTEX 1185 in artificial seawater in a flat panel photobioreactor [42]. Results from this study are therefore promising considering growth in TTWW obviates the need for expensive defined media or other chemical inputs.

The model results were used to ascertain whether factors/variables or interactions between them contributed to microalgal growth (response biomass). The RCM provided highly significant results ($p < 0.0001$) with an adjusted R^2 value of 0.762 indicating that 76.2 % of the variability in the response could be explained by the model. The predicted R^2 value of 0.681 showed that the model predictions were reliable. Table 3 provides the results of the ANOVA for the RCM. Additional statistical data, including regression coefficients and confidence intervals are provided in the Supplementary Material (Table S2).

The biomass concentration was not significantly affected by the $[NH_3]_{initial}$ or the IS ($p > 0.05$) but was significantly affected by the season ($p < 0.0001$) (Fig. 2, Table 3). The GLM results with gamma distribution also indicated that only season had a significant ($p < 0.001$) effect, and that this was true for each season (pairwise comparisons $p < 0.0001$). The results were analysed further to ascertain whether the $[NH_3]_{initial}$ and/or IS affected biomass growth within each season independently. When $[NH_3]_{initial}$ was applied to the GLM as a categorical variable in five concentrations ranges, it was found that $[NH_3]_{initial}$ did have a significant impact on biomass, but only in the autumn/spring simulated runs.

Overall, the biomass growth results indicated that the amount of biomass attained was not affected by the initial amount of biomass added to the flasks. It was also not significantly affected by the $[NH_3]_{initial}$ in the summer- and winter-simulated runs. However, the amount of biomass was highly dependent on climatic factors. In this case, the amount attained was highest in the flasks from the summer-simulated (0.72 ± 0.07 g/L), followed by autumn/spring simulated (0.50 ± 0.11 g/L) and winter-simulated (0.39 ± 0.06 g/L) temperature and photoperiod conditions.

Temperature affects metabolic functions (including CO_2 fixation rates), and growth rates across microalgal species. *Tetradesmus* strains appear to prefer temperatures above 20 °C [43], with optimal temperatures of 22 °C [44], as well 30 °C [45] and 36 °C [43] being reported for

Table 3
Analysis of variance and effect size values for the reduced cubic model for biomass growth.

Source	Sum of squares	df	Mean square	F-value	*p*-value	η^2	
Model	0.7574	8	0.0947	15.03	< 0.0001	0.8165	Significant
A-$[NH_3]_{initial}$	0.0058	1	0.0058	0.9216	0.3456	0.0063	
C-season	0.7050	2	0.3525	55.95	< 0.0001	0.7604	
AC	0.0094	2	0.0047	0.7488	0.4825	0.0101	
A^2	0.0049	1	0.0049	0.7842	0.3837	0.0053	
A^2C	0.0323	2	0.0161	2.56	0.0960	0.0348	
Residual	0.1701	27	0.0063				
Lack of fit	0.1219	18	0.0068	1.26	0.3714		Not significant
Pure error	0.0482	9	0.0054				
Cor total	0.9275	35					

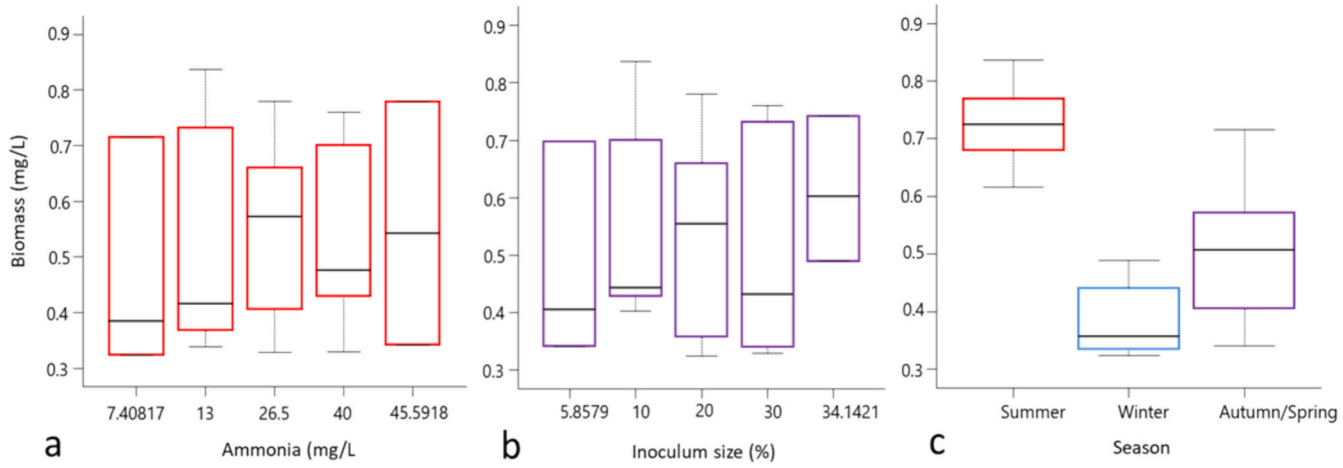

Fig. 2. Box and whisker plots showing the effects of initial ammonia concentration (a), inoculum size (b) and season (c) on biomass growth.

Table 4
Results of: (i) biomass (in %wt.wt, g/kg or mg/kg, as indicated in the table sub-headings) measured in the inoculum (column 8) and at the end of the experimental runs (columns 2–7), and (ii) treated tannery wastewater concentrations (in parentheses in mg/L and μg/L, as indicated in the sub-headings), measured before (column 8) and after (columns 2–7) the experimental runs.

	Winter low	Winter high	Spring/A. Low	Spring/A. High	Summer low	Summer high	Inoculum (TTWW)
Macromolecules (%wt.wt)							
Proteins	32.8	34.9	37.4	33.0	30.6	18.5	26.2
Carbohydrates (GE)	5.5	4.6	8.0	18.5	16.4	22.3	24.9
Fatty acids	3.33	3.80	5.15	6.40	3.70	4.39	5.75
Elemental CHNS (%wt.wt)							
Carbon	39.2	40.7	42.4	45.3	44.5	46.7	40.1
Hydrogen	6.77	7.10	7.48	8.19	8.36	8.77	6.92
Nitrogen	5.67	6.02	6.44	5.69	5.28	31.4	4.52
Major cations (g/kg; mg/L)							
Sodium (Na)	9.47 (797)	10.3 (801)	8.50 (942)	5.51 (808)	6.46 (790)	5.31 (943)	6.54 (997)
Magnesium (Mg)	25.6 (3.7)	24.6 (4.2)	15.4 (5.3)	10.6 (4.5)	18.5 (3.0)	7.81 (4.6)	19.2 (13)
Phosphorus (P)	7.07 (0.1)	7.89 (0.1)	4.80 (0.1)	3.44 (0.1)	4.11 (0.1)	2.59 (0.1)	16.6 (0.3)
Potassium (K)	4.72 (3.9)	3.38 (4.6)	4.39 (4.7)	5.13 (3.1)	4.62 (3.2)	3.53 (3.5)	5.40 (3.3)
Calcium (Ca)	10.2 (17.4)	8.65 (18.7)	9.04 (19.6)	6.48 (16.7)	5.61 (17.9)	2.72 (17.0)	18.3 (18.5)
Minor cations (g/kg; mg/L)							
Silica (Si)	1.65 (2.42)	0.18 (2.52)	0.48 (2.54)	0.60 (1.84)	0.37 (6.56)	0.65 (4.80)	4.39 (0.22)
Minor cations (g/kg; μg/L)							
Aluminium (Al)	4.93 (26.0)	4.15 (31.9)	3.56 (45.9)	2.49 (24.1)	3.61 (69.9)	2.34 (44.5)	0.37 (163)
Manganese (Mn)	16.8 (190)	15.4 (248)	11.6 (288)	8.76 (124)	10.4 (328)	6.64 (379)	7.19 (473)
Iron (Fe)	3.66 (81.8)	1.97 (95.3)	2.74 (95.1)	2.05 (67.4)	2.34 (105)	1.79 (50.0)	2.06 (394)
Zinc (Zn)	0.75 (23.3)	0.69 (32.4)	0.44 (44.0)	0.33 (29.3)	0.41 (35.5)	0.19 (64.5)	0.34 (207)
Minor cations (mg/kg; μg/L)							
Boron (B)	20.8 (272)	15.2 (285)	14.7 (288)	9.14 (194)	34.7 (603)	20.9 (464)	34.5 (44.7)
Vanadium (V)	0.96 (0.2)	0.74 (0.2)	0.82 (0.2)	0.71 (0.1)	0.61 (0.2)	0.58 (0.1)	0.67 (0.13)
Chromium (Cr)	67.6 (22.7)	47.1 (23.6)	50.8 (24.3)	38.9 (20.9)	45.4 (20.7)	34.2 (22.1)	42.6 (29.2)
Cobalt (Co)	1.20 (0.8)	1.16 (1.1)	0.99 (1.1)	0.66 (0.8)	0.87 (0.8)	0.54 (0.8)	1.72 (BDL)
Nickel (Ni)	9.71 (5.9)	7.49 (4.4)	3.78 (7.1)	1.83 (4.3)	2.31 (3.8)	1.65 (4.2)	3.53 (20.6)
Copper (Cu)	35.8 (11.0)	37.3 (7.6)	25.8 (11.7)	35.4 (11.6)	20.8 (5.7)	17.5 (7.3)	22.5 (9.81)
Arsenic (As) (0.1 mg/kg)[a]	0.99 (0.2)	0.94 (0.2)	0.80 (0.2)	0.63 (BDL)	0.59 (BDL)	0.54 (BDL)	0.41 (2.46)
Selenium (Se)	0.33 (0.1)	0.14 (0.2)	0.26 (0.1)	0.22 (BDL)	0.24 (0.1)	0.16 (0.2)	0.13 (BDL)
Strontium (Sr)	31.8 (79.9)	31.8 (82.9)	28.1 (92.4)	20.7 (76.5)	18.9 (78.9)	11.2 (68.1)	65.0 (107)
Molybdenum (Mo)	0.48 (BDL)	0.33 (BDL)	0.44 (BDL)	0.47 (BDL)	0.39 (BDL)	0.37 (BDL)	0.50 (1.2)
Cadmium (Cd) (0.05 mg/kg)[a]	0.15 (0.03)	0.11 (BDL)	0.10 (0.02)	0.06 (0.02)	0.07 (BDL)	0.05 (0.02)	0.04 (0.05)
Tin (Sn) (50)[a]	6.19 (BDL)	1.88 (BDL)	5.52 (BDL)	3.96 (BDL)	5.18 (BDL)	3.72 (BDL)	4.59 (BDL)
Antimony (Sb)	0.28 (0.21)	0.12 (0.24)	0.14 (0.27)	0.16 (0.20)	0.14 (0.19)	0.12 (0.24)	0.19 (0.43)
Barium (Ba)	65.0 (19.4)	58.1 (22.0)	56.1 (22.2)	42.2 (17.2)	44.9 (22.3)	38.1 (18.6)	62.4 (34.8)
Mercury (Hg) (1.2 mg/kg)[a]	0.21 (BDL)	0.31 (5.8)	0.23 (BDL)	0.23 (BDL)	0.17 (3.9)	0.46 (3.8)	0.25 (4.35)
Lead (Pb) (0.05 mg/kg)[a]	6.55 (BDL)	6.06 (BDL)	4.86 (BDL)	4.35 (BDL)	3.48 (BDL)	2.82 (0.2)	3.20 (1.20)

[a] In brackets are upper limit values for edible oil (FAO/WHO, 2021) GE = glucose equivalent; BDL = below detection limit; TTWW = treated tannery wastewater.

strains of *T. obliquus*. Similar to this study, [46] Jo et al. (2020) reported higher biomass productivity and nutrient utilization by a microalgal consortium in a raceway pond in summer (approx. 0.5 g/L) in comparison to spring/autumn (approx. 0.1–0.2 g/L) [46]. *Desdomonas* was the dominant genus, with *Tetradesmus* only constituting around 1 % of the consortium. There are only limited reports on growth of microalgae at temperatures lower than 20 °C. Gao et al. (2023) reported that *Xanthonema hormidioides* adapted to cold stress by accumulating fatty acids and down-regulating photosynthesis, which enabled growth at 5 °C [47]. Christov et al. (2001) also reported reduced chlorophyll and protein concentrations in a strain of *Scenedesmus* when temperatures were reduced from 35 °C to 15 °C. In this study, growth and activity of one or more members of the microalgal consortium at 8 °C is a noteworthy finding, suggesting that phyco-remediation can be applied year-round in climates with moderate winters [48].

3.2. Characterization of biomass for downstream applications

There were seasonal differences in the protein, carbohydrate and fatty acid concentrations of the biomass, but overall trends were consistent (Table 4). Low fatty acid concentrations in all samples precluded the possibility of utilizing the biomass for efficient biodiesel production. This was also noted when the strains were grown individually in TTWW [30]. Higher temperatures and/or photoperiods induced carbohydrate synthesis as the concentrations increased in a continuum from winter to summer. "In general, *Chlorella* spp. and *Limnospira* spp. are known as high protein producing genera (average 60 % and 70 % DCW protein content, respectively) and are commonly used in food, animal and aquaculture feed supplements [49]. In contrast, *Tetradesmus* spp. are considered as oleaginous and suited to the production of biodiesel. However, the low lipid and high protein content of the biomass measured in this study has also been reported by other researchers and is linked to the composition of the growth medium. For example, Cicci et al. (2024) found that *T. dimorphus* produced 47–53 % proteins when grown in olive mill wastewater, while N starvation created via ultrafiltration of the wastewater drove lipidogenesis (49 % DCW lipids) [50]. Likewise, studies with *Neochloris oleoabundans* show that it can accumulate up to 55 % DCW lipids, but in N replete media, the biomass contains up to 55 % DCW proteins [51]. The typically high N content of TTWW therefore appears to lend itself towards the production of high protein microalgal biomass. Pigments and other molecules were not quantified in this study as the intention is to use microalgal consortia in a growth medium (TTWW) and environment (open system) that vary on a temporal basis. Such systems do not lend themselves to producing consistent amounts and types of pigments such as carotenoids. Overall, the high protein and carbohydrate content of the biomass suggested that it could be suitable as a feed supplement year-round, while the favourable N, phosphorus (P), potassium (K) and micronutrient concentrations indicated that it could be applied as an organic fertilizer supplement.

In terms of major cations, the Na, magnesium (Mg), phosphorus (P) and calcium (Ca) were most concentrated in the biomass grown under winter-simulated conditions and least concentrated in the biomass grown under summer-simulated conditions. However, the concentrations of these cations in the bulk liquid of the flasks were similar for each season at the end of the experimental period. This was attributed to the fact that the DCW (biomass) was highest under summer-simulated and lowest under winter-simulated conditions. Some of the minor cations, such as aluminium (Al) and manganese (Mn) increased in the biomass and decreased in the bulk liquid over the experimental period, while the converse was true for others such as silica (Si) and boron (Bo). In terms of toxic metals, Cr was most concentrated in the biomass grown under winter-simulated conditions and the least concentrated in the biomass grown under summer-simulated conditions, although the concentrations in the bulk liquid of the flasks were similar for each season due to difference in biomass concentrations as seen with the major cations. Only negligible changes in the Cr concentrations in the bulk liquid were observed, suggesting that the biomass was already saturated with Cr from long-term acclimation of the microalgal strains in TWW. For tanneries that employ Cr-free tanning, TWW is not expected to contain high concentrations of Cr, although (to the best of our knowledge) there are no other literature reports on Cr concentrations in TWW when Cr-free tanning is employed. The tannery used in this study recently converted from wet-blue to Cr-free tanning, so it is possible that the Cr present in the TTWW emanated from residuals in the wastewater treatment system.

Uptake of arsenic (As), cadmium Cd and Pb from the TTWW took place under all seasonal simulations, with the concentrations being highest in the biomass grown under winter-simulated conditions. In most instances, the concentrations of these toxic metals were higher than those recommended by the Food and Agricultural Organization (FAO) of the World Health Organization (WHO) [52] in edible oil for human consumption, with the concentration of Pb being notably higher (Table 4). Such results suggest that the biomass applications may be limited. Previous results [30] indicated that the biomass of the individual microalgal strains would be suitable as animal or fish feed supplements because of the relatively high protein and carbohydrate contents. However, with the consortia, the risk of Pb entering the food chain may preclude such use, as well as application as an agricultural fertilizer. Further testing is required to establish whether the results were skewed by using microalgal strains that were grown for months in TWW for acclimation purposes and had already accumulated metals within the cells, and whether they would accumulate metals to the same degree when the biomass is continuously harvested.

A possible solution would be to remove toxic metals from the TTWW using chemical or biotic processes upstream of phyco-remediation. Precipitation, ion exchange and electrochemical processes can effectively separate metals, but are usually energy intensive and generate secondary waste requiring specialized disposal [53,54]. Plants, bacteria and fungi can be applied for biosorption, bioaccumulation, or biotransformation [55]. The latter is used to convert metals to less toxic forms, but the oxidation status may revert in the environment. Although economically sustainable, large quantities of biomass contaminated with toxic metals are generated from biosorption and bioaccumulation [54,55].

3.3. Bioremediation of treated tannery wastewater

The composition of the TTWW is provided in parentheses in the last column of Table 4. In addition, the pH was 7.8 and the CODt, CODs, total organic carbon (TOC), VOA and NH_3-N were 876 ± 4, 489 ± 4, 340 ± 37, 101 ± 11 and 36 ± 2 mg/L, respectively ($n = 3$).

3.3.1. Uptake of sodium

As alluded to in Section, 3.2, the Na was most concentrated in the biomass from the winter-simulated runs (9.47 to 10.3 g/kg), and the least concentrated in the biomass from the summer-simulated runs (5.31 to 6.54 g/kg). The concentration of Na in the TTWW (997 mg/L) decreased marginally in all the runs (range: 790–943 mg/L). Due to the consortia being grown historically in high Na TWW, the biomass already contained intracellular Na (6.54 g/kg) so it was not possible to calculate accurate mass balances for Na uptake or indeed any of the other cations. However, considering the average Na concentration in the biomass for each season (Table 4) and average DCW for each season (Section 3.1.1), it was estimated that the consortia with no initial Na in the cells could remove 4.20 mg/L, 3.51 mg/L and 3.86 mg/L Na under summer, autumn/spring, and winter-simulated conditions, respectively. This equates to only 0.42 %, 0.35 % and 0.39 % of the Na in the TTWW used in this study, indicating that phyco-remediation with these halo-tolerant microalgal strains for removal of Na from TWW would not be feasible.

Given the high Na and toxic metals concentrations in TTWW, it is unlikely that many strains would be able to adapt to TTWW and remove sufficient Na. Indeed, the strains used in this study were selected from a

range of marine species that were unable to grow in TTWW. The implications of high Na concentrations in the effluent depends on the site of discharge. If the TTWW constitutes a large fraction of the influent to a municipal wastewater treatment facility, it could impact on the process and/or the quality of the final effluent, while discharging Na via a marine outfall is permissible [56]. Long term discharge to a land environment could result in soil sodicity. In such cases, it would be preferable to remove Na and other ions, using for example, membrane technology [56].

3.3.2. Organics and macronutrients

Phyco-remediation is the process whereby microalgae utilize macronutrients and other contaminants found in municipal and some industrial wastewaters to improve the quality of the final effluent [57]. Microalgal growth may be autotrophic (photosynthetic), chemo-organotrophic (heterotrophic) or mixotrophic [58]. In the presence of light, mixotrophic species grow autotrophically, but can revert to heterotrophic growth when there is insufficient light [58].

In the CCD runs, there were increases in the CODt in all flasks, ostensibly due to *de-nova* photosynthetic carbohydrate synthesis. In comparison to the CODt (Fig. 3a-c), the CODs (Fig. 3d) was relatively stable, decreasing only marginally in the winter- and autumn/spring-simulated runs, and increasing slightly in the summer-simulated runs, possibly due to organic addition via endogenous decay. The measured CODt as well was biomass abundance were lowest in winter (Fig. 3a), increasing through autumn/spring (Fig. 4b) to summer (Fig. 3c). There was a relatively high overall correlation between biomass and CODt (Pearson's $R = 0.795$), while there were moderate correlations in the winter-simulated (Pearson's $R = 0.540$) and summer-simulated (Pearson's $R = 0.479$) conditions, and a low correlation in the autumn/spring-simulated conditions ((Pearson's $R = 0.369$).

This could be explained by the fact that autotrophic growth is expected to be highest in summer when the photoperiod is longest and lowest in winter when the photoperiod is shortest. Conversely, longer dark cycles should theoretically support more heterotrophic growth with concomitant CODs utilization. Likewise, heterotrophic metabolism should be more marked in winter and the different seasonal balances between autotrophic growth and heterotrophic CODs utilization may explain the study results because *T. obliquus* and a variety of *Neochloris* spp. have been shown to be capable of both autotrophic and heterotrophic growth [6,7]. In contrast to a previous study where VOA accumulated in cultures of *Neochloris* sp. CPUT-W1 and *T. obliquus* CPUT-L1 [30], respectively, the VOA concentrations decreased from the influent value of 105 mg/L by >50 % in all the flasks, indicating improved VOA utilization when the strains were present as a consortium (Fig. 3d).

The concentrations of soluble SO_4 in the TTWW (1020 mg/L) decreased by averages of 8 %, 17 % and 8 % in the winter, autumn/spring and summer-simulated flasks, respectively, with final respective concentrations of 933 ± 30 mg/L, 848 ± 84 mg/L and 941 ± 44 mg/L. The higher utilization in spring/autumn-stimulated conditions, despite attaining less biomass than in the summer-simulated conditions may be linked to higher heterotrophic metabolism in autumn/spring dependent on both light and temperature. Indeed, Pan et al. (2023) demonstrated up-regulation of genes encoding sulfur (S) uptake in *Chlorella vulgaris* under dark growth conditions [59]. Together with less stable NH_3 utilization patterns (Fig. 5), these results suggest that there was less stability in the growth and metabolism of the consortia under the autumn/spring-simulated light and temperature conditions.

The TP was consistently <1 mg/L, but TP concentrations in TWW are expected to be low, and removal of P is not a goal of biological TWW treatment [60]. On the contrary, P is sometimes added to balance microbial nutrient requirements in secondary treatment systems [60]. No

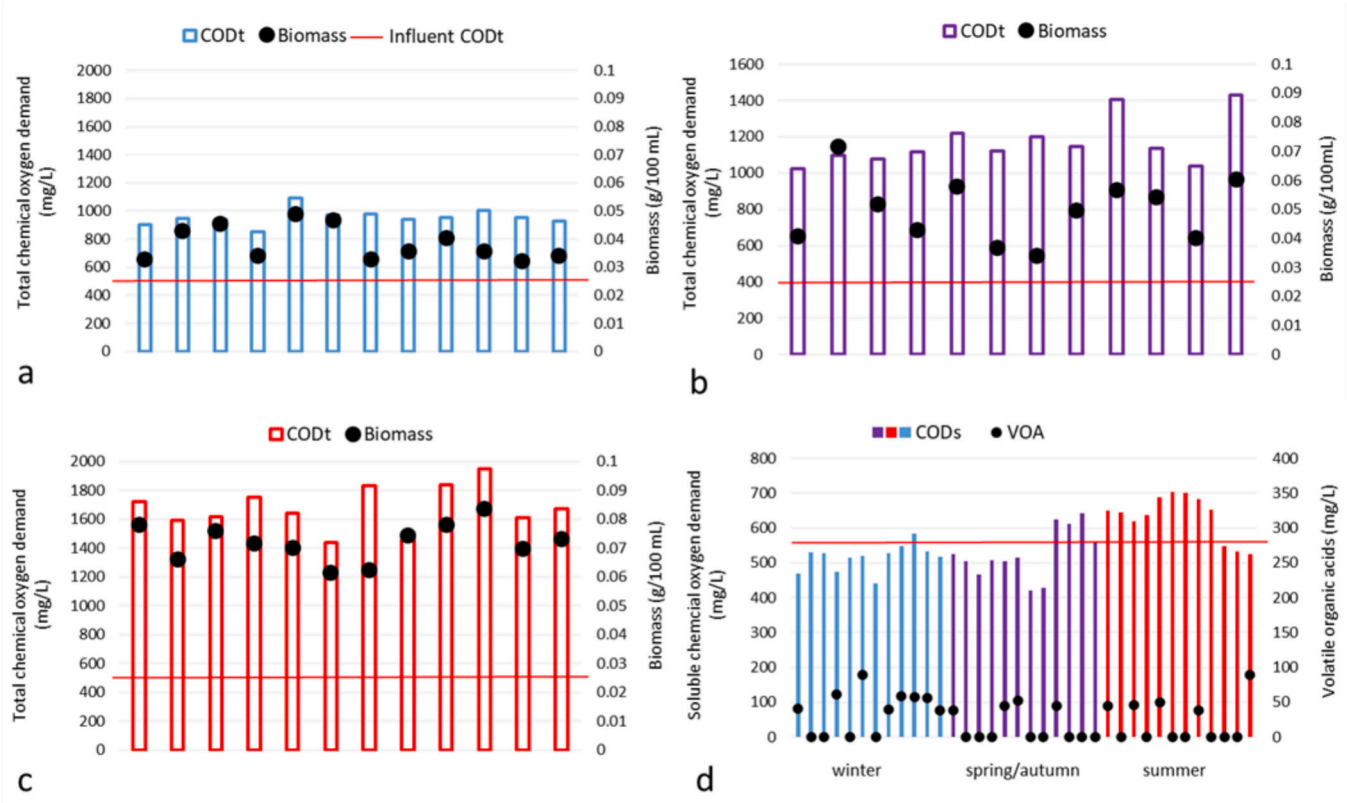

Fig. 3. Comparison between the total chemical oxygen demand and biomass measurements at the end of the experimental period in the winter (a), spring/autumn (b) and summer (c) simulated experiments, and comparison between the soluble chemical oxygen demand and volatile organic acid concentrations during winter, spring/autumn and summer (d). The initial chemical oxygen demand concentrations are indicated on the figures by red horizontal lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

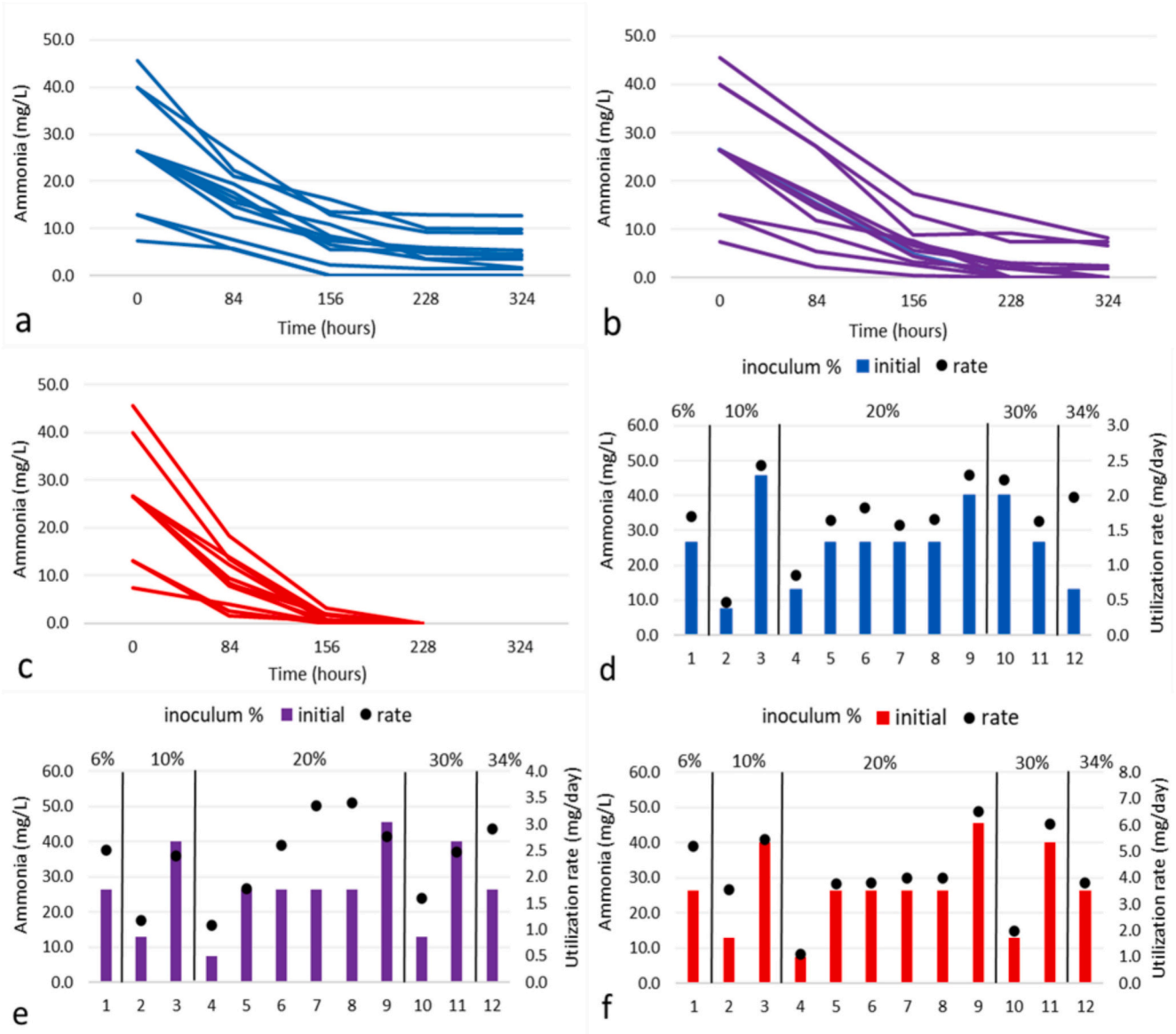

Fig. 4. Temporal decreases in the ammonia concentrations in the winter (a), autumn/spring (b) and summer (c) simulated experiments, and relationships between the initial ammonia concentration, ammonia utilization rate and initial inoculum size in the winter (d), autumn/spring (e) and summer (f) simulated experiments. The initial inoculum sizes (%) are provided at the top of Figures d-f and delineated by black vertical lines.

in-depth analysis of TP removal was therefore required for this study.

3.3.3. *Ammonia utilization*

In the study, NH_3/NH_4 was the major contributor to TN and other forms of N were therefore not measured. In conventional secondary wastewater treatment systems, NH_3 is utilized by nitrifying bacteria as a source of electrons for reducing NH_3 to nitrite (NO_2^-). In contrast, microalgae take up NH_3 to synthesise pigments, proteins and nucleic acids [24].

The NH_3 utilization patterns were consistent with those of microalgal growth from winter through to summer. There were temporal pH increases over the first 156 h (from initial 8.5 ± 0.17, 8.3 ± 3 0.03 and 8.7 ± 0.18 in the winter- spring/autumn and summer-simulated runs, respectively), corresponding to decreases in NH_3 concentrations. In the winter- and spring/autumn-simulated runs, the pH continued increasing (to 10.2 ± 0.29, and 10.2 ± 0.26, respectively) and NH_3 concentration decreasing (Fig. 4a,b,d,e) until the end of the experimental period. In the summer-simulated runs, the pH increased (to 9.9 ± 0.48) over the first 156 h (6.5 days) and then stabilized. In addition, NH_3 utilization was complete in all the summer-simulated runs by 228 h (9.5 days) (Fig. 4c). In contrast, complete NH_3 utilization only occurred in 17 % and 42 % of the winter and spring/autumn-simulated runs, respectively, clearly demonstrating the positive effect of increased temperature and/or photoperiod on NH_3 utilization. The T.alk. reduced from 156 mg $CaCO_3$/L to 83 ± 15, 104 ± 19 and 118 ± 24 mg $CaCO_3$/L in the winter-, spring-autumn- and summer-simulated flasks, respectively. Previously, initial decreases in T.alk. due to NH_3 uptake were followed by increases ascribed to fixation of CO_2 and possible proton uptake [30,61]. In this study, it was assumed that the higher T.alk in the summer-simulated through to the winter-simulated runs was due to increased CO_2 fixation correlated with higher temperatures and/or photoperiods. This is supported by the CODt results that also increased from 'winter' through to 'summer'.

It should be noted that some NH_3 losses due to volatilization may have taken place as the pKa of NH_3 is 9.24 under standard conditions (25 °C, 1 atm.). However, the pH values were lowest in the summer-simulated runs which exhibited higher NH_3 utilization, ruling out volatilization as a significant contributor to NH_3 removal.

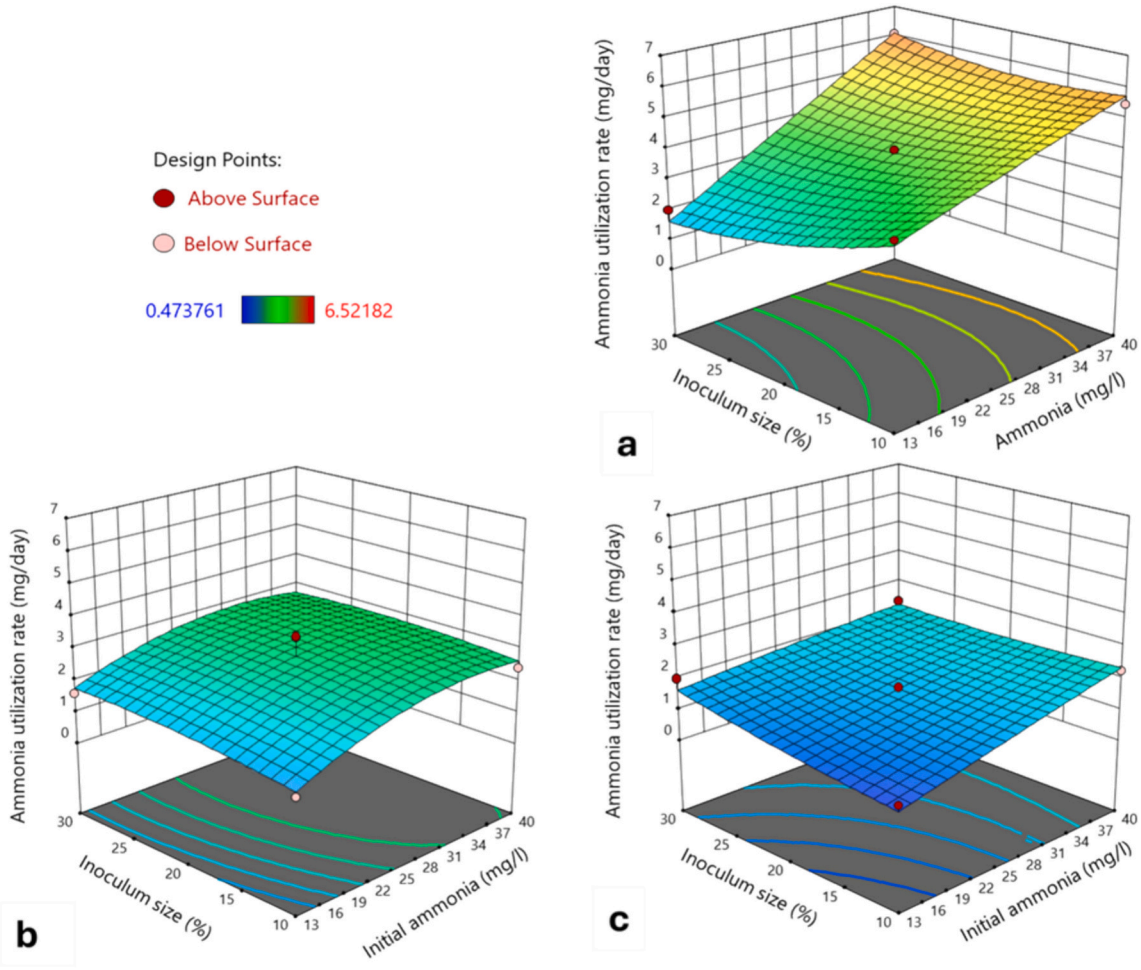

Fig. 5. Response surface methodology plots showing the effects of the initial ammonia concentration on the ammonia utilization rate under summer (a), autumn/spring (b), and winter (c) conditions.

There were highly significant correlations ($p < 0.001$) between the $[NH_3]_{initial}$ and the NH_3 utilization rate for each simulated season (Fig. 4d-f), with high Pearson's correlation coefficients in the summer- ($R = 0.932$) and winter- ($R = 0.843$) simulated experiments, and a moderately high co-efficient in the autumn/spring-simulated experiments ($R = 0.619$).

Table 5 provides the results of the ANOVA with non-significant terms eliminated for the RCM for NH_3 utilization. Additional statistical data, including regression coefficients and confidence intervals are provided in the Supplementary Material (Table S3). The results for the RCM and GLM both confirmed that $[NH_3]_{initial}$ and season played significant ($p < 0.0001$) roles in the NH_3 utilization rate while the IS on its own did not ($P > 0.5$). The effect of season on the NH_3 utilization rate was highly significant for all seasons (pairwise comparisons $p < 0.001$). There were also significant interactions between $[NH_3]_{initial}$ and season on the NH_3 utilization rate. The effect of $[NH_3]_{initial}$ on NH_3 utilization was more pronounced in the summer-simulated runs. Overall, the model predicted that the highest NH_3 utilization rate could be obtained with an IS of 30 % under summer-simulated conditions with a $[NH_3]_{initial}$ of 40 mg/L.

In comparison to the CCD experiment, it took longer for the $[NH_3]_{initial}$ to be utilized in the 1 L (600 mL working volume) flasks used to harvest biomass for characterization. To reach similar end points to the CCD experiment, the 1 L flasks were incubated for 468 h (19.5 days) as opposed to 324 h (13.5 days) for the 500 mL flasks. The CODt was marginally higher in the 600 mL 'low' biomass runs than in the 'high" biomass runs under spring/autumn-simulated conditions, but similar trends were noted for CODt and biomass in the other runs irrespective of volume (Fig. 6a-b). Both CODt and biomass were generally higher in the

Table 5
Analysis of variance and effect size values for the reduced cubic model for ammonia utilization.

Source	Sum of squares	df	Mean square	F-value	p-value	η^2	
Model	66.41	6	11.07	41.79	<0.0001	0.8964	Significant
A-$[NH_3]_{initial}$	21.14	1	21.14	79.84	<0.0001	0.2853	
C-season	37.48	2	18.74	70.77	<0.0001	0.5058	
AC	6.89	2	3.44	13.00	<0.0001	0.0930	
^{2}	0.8927	1	0.8927	3.37	0.0766	0.0120	
Residual	7.68	29	0.2648				
Lack of fit	5.90	20	0.2948	1.49	0.2758		Not significant
Pure error	1.78	9	0.1982				
Cor total	74.09	35					

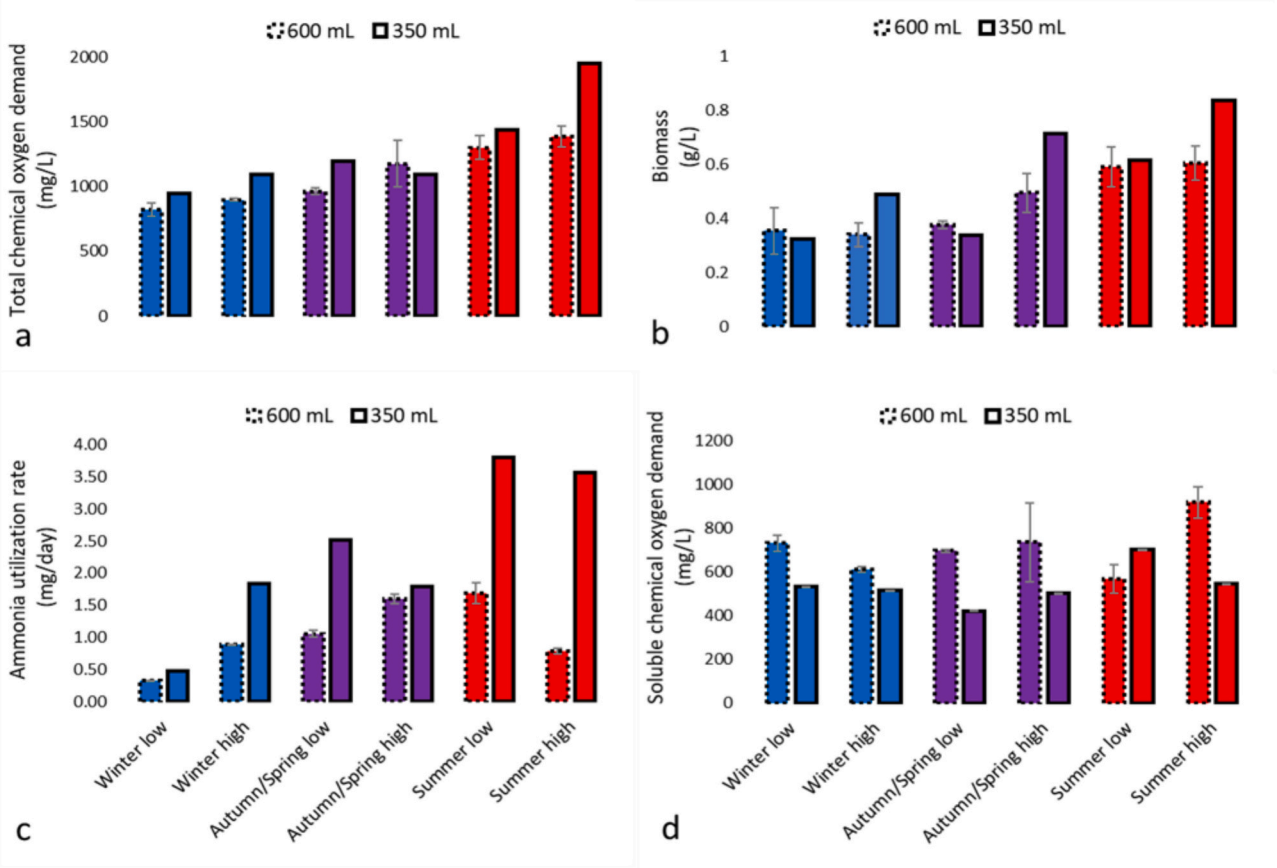

Fig. 6. Comparison of total chemical oxygen demand (a), biomass (b), ammonia utilization rate (c) and soluble chemical oxygen demand results measured at the end of the 350 mL and 600 mL experimental runs (blue = winter, purple = autumn/spring, red = summer). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

350 mL runs despite being conducted for a shorter period. It was postulated that the decreases were due to additional shading by the microalgae themselves at the higher volume as described by Soroosh et al. (2022) [62] and/or differences in aeration due to smaller surface to volume ratios and higher volumes in the larger flasks that decreased the mass transfer of atmospheric gases into the liquid phase [63]. In most runs, the NH_3 utilization rates were lower (Fig. 6c). The CODs were high (Fig. 6d) in the 600 mL flasks, clearly indicating that both the growth and metabolic functions of the consortium were affected by marginally increasing the volume. Indeed, researchers have cautioned against extrapolating findings from flask studies and smaller-scale reactors to pilot and full-scale systems [64], although a model has been successfully validated for scaling-up airlift photobioreactors [65]. Results from flask studies do, however, provide fundamental information from multiple experiments that would not otherwise be logistically possible. Such data provides important information required for setting initial operating parameters in bioreactors and to indicate whether processes may or may not be feasible (proof of concept studies).

4. Conclusions and future work

The microalgal consortium was able to successfully remove NH_3 but not Na from the TTWW, and the NH_3 removal rates were dependent in the $NH_{3initial}$ and simulated climatic conditions. The highest biomass (0.780 g/L) and NH_3 utilization (6.52 mg/day) were obtained under summer-simulated climatic conditions (27 °C, 14 h light) at the highest $[NH_3]_{initial}$ applied in this study (45.59 mg/L). The results indicated that microalgal-TTWW biorefineries would be most appropriate in countries with warm climates treating TTWW with $[NH_3]_{initial}$ sufficient to stimulate growth. In terms of biorefinery applications, there were insufficient fatty acids for biodiesel production. In this study, the levels of Pb were higher than those recommended by the WHO for human consumption, but results may have been skewed by long-term acclimation of the strains to TWW. Long term studies are therefore required to assess levels of toxic metals such as Pb in the biomass of continuously operated systems. The high protein and carbohydrate content, as well as the presence of micronutrients suggest that the biomass could be utilized for animal or fish feed supplementation provided the levels of toxic metals are acceptable. Alternatively, the biomass, with suitable N P and K content, could be used as an organic agricultural fertilizer supplement.

Many studies focus on optimizing microalgal growth conditions that are unrealistic for full-scale implementation. While fundamental knowledge contributes valuable information, this study showed the importance of studying microalgal growth and remediation under climatic conditions likely to be expected in the geographical area where systems will be installed. Building on the results of this study, further pilot-scale research on microalgal-bacterial (MAB) remediation systems is required because: (i) it is not feasible to prevent growth of other microbes in full-scale systems, and (ii) removal of organic molecules is dependent on heterotrophic bacterial growth because microalgae can increase the CODt via fixation of CO_2.

CRediT authorship contribution statement

A. Ranjan: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **P.J. Welz:** Writing – review & editing, Writing – original draft, Visualization, Validation,

Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104137.

Data availability

Data will be made available on request.

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