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Valorisation of tannery wastewater with indigenous microalgal strains: Acclimation, identification, biomass characterization and remediation

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ABSTRACT

Tannery wastewater contains high concentrations of inorganic and organic pollutants and requires extensive remediation before discharge. In this study, four of ten promising microalgal strains were successfully acclimated to 50 % raw tannery effluent. Three of the strains were indigenous species that were identified using molecular techniques and designated as *Tetradesmus obliquus* CPUT-L1, *Tetradesmus dimorphus* CPUT-L2 and *Neochloris* sp. CPUT-W1. The biomass concentrations (1.50 ± 0.017 to 1.11 ± 0.04 g/L) compared well with literature values for growth of microalgae in wastewater. Growth in the nitrogen-rich tannery effluent drove the metabolism of all four strains to produce higher amounts of carbohydrates (12.7–19.8%wt.wt.) and proteins (24.5–37.2%wt.wt.) than fatty acids (7.40–13.3%wt.wt.). Results suggest that the high-protein biomass could be used as an animal feed supplement because all samples also contained a range of micronutrients, and the concentrations of toxic metals were compliant with the World Health Organization standards for human consumption. All strains were able to completely remove ammonia (12 mg/L) from the effluent. The concentrations of soluble COD (489 ± 4 mg/L) did not change significantly after remediation and there were notable increases (64–89 %) in total COD from influent (876 ± 4 mg/L) to effluent due to autotrophic microalgal growth. All the strains formed large coenobia, allowing the biomass to settle readily without the need for physical or chemical interventions. Overall, the results showed that the acclimated microalgal strains have the potential to be used in a tannery biorefinery for simultaneous polishing of tannery effluent and possible utilization of the biomass as animal feed.

1. Introduction

Microalgae can utilize macronutrients and other potential environmental contaminants in municipal and industrial wastewater for growth [1]. Phyco-remediation is the process whereby the quality of the effluent is improved by the growth and metabolic activity of the microalgae. Microalgal growth may be autotrophic (photosynthetic), heterotrophic or mixotrophic [2]. Mixotrophic species grow autotrophically in the presence of light and heterotrophically in the absence of light [2]. In the move towards a circular economy, microalgal wastewater biorefineries are being adopted to simultaneously remediate wastewater and produce microalgal biomass for downstream applications such as biodiesel production, animal feed and biofertilizer [3,4,61].

Tannery wastewater (TWW) from beamhouse processing is typically saline, has a high organic load, and contains metals. Laboratory studies suggest that phyco-remediation and valorisation of TWW is possible [5,6], ostensibly because some strains of microalgae produce protective enzymes that enable them to proliferate in extreme metal- and salt-containing environments [7]. Microalgae have been successfully grown in TWW in laboratory environments when TWW was diluted [8,9], mixed with sewage [10], after primary pre-treatment via ozonation [11,12] or after secondary treatment [13]. Arutselvan et al. [14] were able to grow microalgae in 'raw' TWW, but the aetiology of the batch of TWW used in the study was not provided and the organic load indicators (chemical oxygen demand (COD) and/or biological oxygen demand (BOD) were not measured. It may be assumed that the batch of TWW was only marginally toxic or non-toxic because the concentration of sulfates (SO_4^{2-}) in the raw TWW was low (254 mg/L) in comparison to values typically measured in beamhouse effluent (2865 ± 796 mg/L, $n = 6$, [15]). Overall, the results from previous studies suggest that phyco-remediation may be suitable as a tertiary (polishing) rather than a secondary step for treatment and valorisation of TWW.

Microalgal genera that have been shown to proliferate in TWW include Chlorella, Scenedesmus, Coelastrella, Clorococcum, Hapolosiphon, Nannochloropsis, Thalassiosira, Dunaliella, Tetraselmis, Naannochloropsis and Pseudochlorella ([8,11,12,13,14]; Saranya &

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Shanthakumar, 2022a, 2022b; [9]). Studies have indicated that strains of Chlorella and Scenedesmus/Tetradesmus grown in TWW may be suited to energy production through esterification for biodiesel production [9,13,14] or anaerobic digestion (AD) [16,17].

This study focusses on the growth, remediation potential and biomass characteristics of one commercial and three indigenous strains of microalgae that were successfully acclimated to diluted TWW. The isolates were identified using microscopic and molecular methods and their potential for tertiary remediation of treated TWW (TTWW) was evaluated. The dried biomass was characterized for potential downstream applications.

2. Materials and methods

2.1. Microalgal strains

2.1.1. Microalgal strain selection

Seven halotolerant microalgal strains (*Botryococcus braunii* CCAP 807/2, *Chlorella protothecoides* var. communis CCAP 211/10C, *Tetradesmus obliquus* CCAP 276/1 A (formerly *Scenedesmus basiliensis* CCAP 276/1 A), *Spirulina major* CCAP 1475/3, *Chlorella gloriosa* CCAP 211/25, *Nannochloropsis oceanica* CCAP 849/10 and *Tetraselmis suecica* CCAP 66/4) were purchased from the Culture Collection of Algae and Protozoa (CCAP), UK. In addition, indigenous microalgal strains were harvested from: (i) the liquid phase in laboratory bottles containing TWW, where they grew spontaneously, and (ii) the liquid phase at the top of a biosand reactor treating winery wastewater [18], where they also grew spontaneously. All strains were screened for the ability to proliferate in TWW by firstly growing in defined bold basal medium (BBM) with increasing concentrations of raw TWW. None of the strains were able to grow in 100 % raw TWW, but they all proliferated in 10–20 % raw TWW [15]. Subsequently, acclimatization and densification of the cultures in 30–50 % raw TWW was attempted over an eight-month period. Only *T. obliquus* CCAP 276/1 A and three indigenous strains (CPUT-L1, CPUT-L2, CPUT-W1) were successfully acclimated to growth in 50 % raw TWW. These four strains were selected for further studies.

2.1.2. Identification of selected indigenous microalgal strains

The selected strains were examined microscopically at the Scientific Services laboratory (City of Cape Town) and the Central Analytical Facility (CAF) of Stellenbosch University (Stellenbosch, South Africa) using conventional light microscopy and scanning electron microscopy (SEM), respectively. The SEM was conducted according to the method described by Sehgal et al. [19] using a Zeiss (Oberkochen, Germany) MERLIN Field SEM microscope. Light microscopy was conducted under 1000× magnification using oil immersion.

For molecular identification, deoxyribonucleic acid (DNA) was extracted from 0.5 g biomass from each selected indigenous strain using a Qiagen (Hilden, Germany) DNeasy Powerlyzer PowerSoil DNA isolation kit according to the manufacturers' instructions. The purified DNA from each of the strains was amplified using the primer pair sets (Integrated DNA Technologies, Inc. (IDT), Coralville, IA, USA) shown in Table 1. The concentration and quality of the extracted genomic DNA were measured spectrophotometrically with a NanoDrop 2000 (Thermo Fisher Scientific, Boston, MA, USA). For PCR amplification, 100 ng of each isolate was used in a 50 μL reaction that contained 25 μL of 2 x DreamTaq polymerase (Thermo Fisher Scientific), 0.1 μM of each primer and nuclease-free water. Thermal cycling consisted of an initial denaturation step at 95 °C for 10 min, followed by 40 cycles of 1 min at 95 °C denaturation, 1 min at 52 °C (L1, L2 and W1 using D512/978 rev); 1 min at 55 °C (L1 and L2 with ITS1/4; L2 with Diat-rbcL-F/iR); 1 min at 57 °C (L1 with Diat-rbcL-iF/R) and 1 min at 60 °C (W1 with ITS1/4) annealing step respectively and 1 min at 72 °C elongation. The PCR products were visualized in 1 % agarose gel. The PCR products was purified using Machery-Nagel™ (Düren, Germany) Nucelospin™ Gel and PCR purification kit and stored at −20 °C until further analysis.

Table 1
Forward and reverse primers used for identification of indigenous microalgal strains.

Primers	Nucleotide sequence (5′-3′)	Reference
F: D512	ATT CCA GCT CCA ATA GCG	[20]
R: D978 rev	GAC TAC GAT GGT ATC TAA TC	
F: Diat-rbcL-F	AAG TGA GCG TKA CGA ATC TGG	[21]
R: Diat-rbcL-iR	AAG AAW CGY TCT CTC CAA CG	
F: Diat-rbcL-iF	AAG GHT TAA AAG GTG GTT TAG A	[21]
R: Diat-rbcL-R	RTA RAA ACC TTTAAT CA	
F: ITS1	TCC GTA GGT GAA CCT GCGG	[22]
R: ITS4	TCC TCC GCT TAT TGA TATGC	

F = forward, R = reverse.

The amplicons were sequenced at the Central Analytical Facility (CAF) at Stellenbosch University (SU) (Stellenbosch, South Africa) using an Applied Biosystems (Waltham, MA, USA) ABI3730xl instrument. The sequences were analysed using DNAMAN version 10.0.2.100 (Lynnon Biosoft, San Ramon, CA, USA). Sequences were compared to those in the GenBank database using the National Centre for Biotechnology Information (NCBI) basic logical alignment search tool (BLAST) for homologous analysis. Sequences were deposited in the NCBI database under Project PRJNA1172546.

For the construction of the phylogenetic trees, the evolutionary history was inferred by using the Maximum Likelihood method (with 100 bootstrap replicates) and the Tamura-Nei model [23], whereby the sequences were aligned using the multiple sequence alignment tool in the ClustalW software package. Sequence alignment as well as tree building and evolutionary analyses were performed using MEGA11 [24]. For construction of the phylogram for both D512 and ITS sequences, *Elliptochloris bilobate* was used as an outgroup. Initial tree(s) for the heuristic search were obtained by applying the neighbor-Joining method to a matrix of pairwise distances estimated using the Tamura-Nei model.

2.2. Experimental procedures

The four microalgal strains were grown individually in 100 % TTWW, the characteristics of which are presented in Table 2. The TTWW consisted of mixed beamhouse and tanyard effluent that had undergone primary settling and secondary remediation. The TTWW was autoclaved before use to exclude other microorganisms that could influence the results. No hydrogen sulfide (HS^-) was detected in the TTWW and no nutrients were added. The stains were grown in 500 mL flasks (350 mL working volumes) at 21 °C in shaking incubators at 160 rpm with a photoperiod of 16:8 (light:dark cycles). Light was provided using 17 W 31 μmol/s Sylvania (Budapest, Hungary) Gro-Lux LED grow bulbs. Initial growth cycles of 60–70 days were measured, with exponential phases of approximately 25 days and stationary phases of 30–35 days. After further acclimation, the growth cycles were reduced to 30–35 days, with exponential phases of 15 days. Final experiments (the results of which are presented in this manuscript) were conducted in duplicate using (i) individual strains at 20 % inoculum density using the same batch of TTWW under the same conditions as previously for 58 days, and (ii) a consortium of all four strains with equal amounts of biomass (g/L) of each at 20 % inoculum density for 14 days under conditions mimicking the average temperature (27 °C) and photoperiod of 14:10 (light:dark cycles) for summer in Cape Town, South Africa.

2.3. Sampling and analytical procedures

2.3.1. Wastewater samples

Samples of flask contents (2 mL) were taken aseptically every 3 to 4 days for measurement of total alkalinity (T.Alk), pH, and ammonia (NH_3). The other chemical parameters were measured at the end of the experimental period: total and soluble chemical oxygen demand (CODt,

Table 2
Chemical characteristics of treated tannery wastewater in mg/L except for pH (units) and T.Alk (mg $CaCO_3$/L) ($n = 2$).

pH	CODt	CODs	TOC	VOA	TN	NH_4^+	T.Alk	TP	SO_4^{2-}	Cl^-
7.8	876 ± 4	489 ± 4	340 ± 37	101 ± 11	36 ± 2	7.4 ± 0.4	162 ± 3	0.3 ± 0.04	1040 ± 85	1975 ± 64

CODt = total chemical oxygen demand; CODs = soluble chemical oxygen demand; TOC = total organic carbon; VOA = volatile organic acids; TN = total nitrogen; NH_4^+ = ammonium; T.ALK = total alkalinity; TP = total phosphorus; SO_4^{2-} = sulfate; Cl^- = chloride.

CODs), total organic carbon (TOC), sulfate (SO_4^{2-}), sulfide (S^{2-}), total nitrogen (TN), total phosphate (TP), and volatile organic acids (VOA). The pH, CODt, TN and TP were performed on whole samples, while the other analyses were conducted on filtered samples (soluble fractions). The pH was measured using a Eutech (Eutech instruments, Singapore) pH 700 instrument and pH probe. The chemical analyses were conducted on the TTWW influent and the samples from the flasks using a Merck (Darmstadt, Germany) Spectroquant Pharo instrument) together with standards and controls as per the manufacturers' instructions as previously described [25].

2.3.2. Biomass samples

The biomass was dried in an oven at 60 °C overnight, crushed to a fine powder using a pestle and mortar and characterized. Major and minor elements were quantified 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 at the Central Analytical Facility (CAF) at Stellenbosch University (Stellenbosch, South Africa) after extraction of 0.5 g the dried microalgal powder in 6:1 (vol.vol) nitric acid (HNO_3) and hydrogen peroxide (H_2O_2) in a MARS (CEM corporation, Matthews, NC, USA) microwave digestion system at 210 °C (ramp time of 20 min, hold time of 10 min). The carbon, hydrogen, nitrogen, sulfur (CHNS) content (%wt/wt) in the digestate was determined using an Elementar (Hamburg, Germany) Vario EL cube Elemental analyser according to the manufacturers' instructions, also at CAF. The protein content was determined by multiplying the N content by a factor of 5.8 [26]. The fatty acids methyl esters (FAME) were measured after digestion of the dried microalgal powder in 2 % sulfuric acid (H_2SO_4) in methanol for 40 min at 80 °C, extraction into diethyl ether ($(C_2H_5)_2O$) and petroleum ether, removal of the upper layer and reconstitution in 1:1 (vol.vol) $CHCL_3$ and $(C_2H_5)_2O$, followed by derivitization/methylation in 2 % sulfuric acid (H_2SO_4) in methanol and toluene (2:1 vol.vol) for 45 min at 100 °C and extraction in 20 % hexane. The FAME were then qualitatively and quantitatively evaluated 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. The carbohydrate content was determined using the Anthrone spectroscopic method after alkaline extraction with bead beating as described by Chen and Vaidyanathan [27].

2.3.3. Microalgal growth profiles

The temporal changes in biomass were measured using chlorophyll-a as a proxy by obtaining the optical density (OD) at 680 nm. Samples of flask contents (2 mL) were taken aseptically every 3 to 4 days for OD measurements. At the end of the study, gravimetric biomass measurements were conducted on dried biomass obtained from the flasks and dried at 60 °C for 2–3 days. Standard graphs were constructed from chlorophyll-a measurements of serial dilutions of the dried biomass (Fig. 1) and used to convert the temporal chlorophyll-a measurements into g/L.

3. Results and discussion

3.1. Indigenous microalgal strain identification and evaluation

Due to the limited range of morphological features and the variability in phenotypic traits among algal species, these characteristics are not sufficiently reliable for identification and may lead to misinterpretation, particularly when comparing samples from different environments [28,29]. To address this issue, genetic identification of microalgae based on the analysis of specific genomic sites has become a valuable molecular tool for reliable species differentiation [28,30]. Common

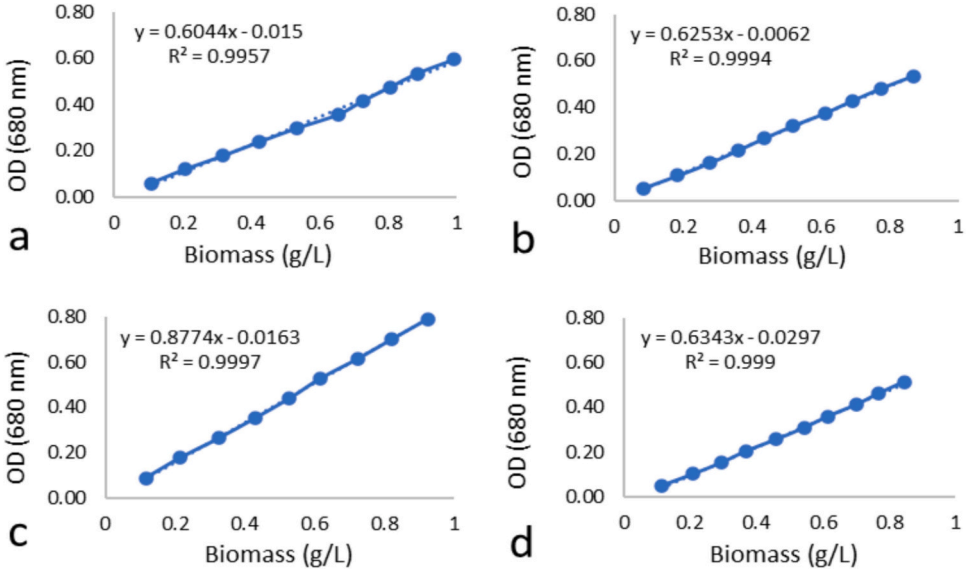

Fig. 1. Standard plots of dried biomass plotted against optical density for *Tetradesmus obliquus* CPUT-L1 (a), *Tetradesmus dimorphus* CPUT-L2 (b) *Neochloris* sp. CPUT-W1 (c) and *Tetradesmus obliquus* CCAP 276/1A (d).

regions used for taxonomic differentiation of microalgal species are the 18S rDNA, D512 region, internal transcribed spacer (ITS) and rbcL regions, due to their conserved nature and high sensitivity [19,28].

The 18S rDNA region is highly conserved and widely used for broad taxonomic identification; however, effectiveness for distinguishing closely related species is limited. In contrast, the D512 region offers greater variability, making it more suitable for differentiating closely related species, though its use may be constrained by the availability of reference sequences. The ITS regions offer high variability, making them proficient for species-level identification. The primary advantage of using ITS regions is their high resolution, which allows for clear differentiation between closely related species. The rbcL region, although useful for identifying broad taxonomic groups in photosynthetic organisms, has low variability, which makes it less effective for distinguishing closely related species [19,28].

In this study, the ITS region of their nuclear genome, the rbcL gene of the chloroplast genome as well as a segment of the 16S ribosomal RNA gene were amplified and NCBI nucleotide BLAST was run to study their sequence similarity with species already reported in the GenBank database. The amplicon lengths, accession numbers, and closest identifiable matches are presented in Table 3.

The phylogenetic relationships of the isolates and other reported homologs based on their specific regions are presented in phylogenetic trees. For the D512 region (Fig. 2), the analysis involved 24 nucleotide sequences with a total of 2450 positions in the final dataset. The CPUT-L1 and CPUT-L2 microalgal strains clustered together with several *Tetradesmus* and *Scenedesmus* spp. with a similarity of 99 % and CPUT-W1 formed a group with *Neochloris* sp. AB917132.1 with a bootstrap value of 80 %. The ITS region showed similar relationships among the isolates (Fig. 3).

For the ITS region, the analysis involved 19 nucleotide sequences with a total of 2478 positions in the final dataset. The CPUT-L1 microalgal strain grouped with *Tetradesmus obliquus* and the CPUT-L2 microalgal strain formed a clade with *Scenedesmus dimorphous*. However, the systematics of the family *Scenedesmaceae* has changed since the advent of more sophisticated molecular identification methods [28]. *Scenedesmus obliquus* was first reassigned as *Acutodemus obliquus* and then (currently) as *T. obliquus* (Oliveira et al. [31]). The microalgal strain CPUT-W1 clustered with *Neochloris conjuncta*. Interestingly, at the start of the project, the commercial strain of *Tetradesmus obliquus* (CCAP 276/1 A) that acclimated to TWW was purchased as *Scenedesmus basiliensis* CCAP 276/1 A and has subsequently been taxonomically reassigned.

The primers used in this study for the rbcL region of the isolates could not amplify in the CPUT-W1 isolate. For the rbcL region, the CPUT-L1 and CPUTL2 isolates also grouped within the *Tetradesmus* genus (Supplementary material Fig. S1).

In summary, using robust molecular methodology, the isolated strains CPUT-L1, CPUT-L2 and CPUT-W1 examined in this study were closely related to *T. obliquus*, *Tetradesmus dimorphus* and *Neochloris* sp., respectively, and were putatively named *T. obliquus* CPUT-L1, *T. dimorphus* CPUT-L2 and *Neochloris* sp. CPUT-W1, respectively.

T. obliquus has been researched for a wide variety of industrial applications, with over 2000 related literature articles [31]. In comparison, there is less information on *T. dimorphus and Neochloris*. The microscopic morphologies of the respective indigenous study strains (Fig. 4) were consistent with previous descriptions of *T. obliquus* ([28,31–35]), and *Neochloris* spp. [36], although the cells of *T. dimorphus* CPUT-L1 were less spindle-shaped than those in the images of *T. dimorphus* FBCC-A330 provided by Cho and Lee [28].

It has been reported that the cell walls of *T. obliquus* are composed of three layers (an inner high density mature cell wall, a cellulosic layer and an outer trilaminar sheath), and the cells contain single cup-shaped chloroplasts [32]. These were all clearly visible in the *T. obliquus* CPUT-L1 isolate. Longitudinal waves or hems visualized in *T. obliquus* IPPAS-2023 [33] were also noted in *T. obliquus* CPUT-L1. The cells of *T. obliquus* CPUT-L1 and *T. dimorphus* CPUT-L2 ranged from 5.4 μm to 7.9 μm and 4.9 μm to 5.4 μm, respectively, consistent with ranges reported in literature [31,33], although large size variations have been ascribed to differences in environmental conditions, including light intensity [35]. During initial isolation in synthetic medium (Section 2.1), the indigenous strains grew in single forms and/or tetrads. After growth in TTWW, the cells of all the strains were grouped in large coenobia, shown for *T. obliquus* CPUT-L1 and *Neochloris* sp. CPUT-W1 in Fig. 4e

Table 3
Designation and accession numbers of indigenous microalgal strains and details of the closest relatives identified with the three different primer sets.

Designation NCBI Accession number	CPUT-L1 SAMN44272498	CPUT-L2 SAMN44272499	CPUT-W1 SAMN44272500
Family	*Scenedesmaceae*	*Scenedesmaceae*	*Neochloridaceae*
Class	*Chlorophyceae*	*Chlorophyceae*	*Chlorophyceae*
Genus	*Tetradesmus*	*Tetradesmus*	*Neochloris*
D512 sequence comparison			
Accession number	MT613871.1	OR551482.1	AB917132.1
Length (bp)	388	388	391
Closest relative	*T. obliquus* CABeR42	*T. dimorphus*	*Neochloris* sp.
% similarity	99	99	92
ITS sequence comparison			
Accession number	MT270137.1	AJ400488.1	KM020061.1
Length (bp)	243	262	380
Closest relative	*T. obliquus* UTEX72	*S. dimorphus* UTEX 417[a]	*N. conjuncta* SAG 78.80
% similarity	99	100	70
rbcL sequence comparison			
Accession number	CP126192.1	MG969380.2	[b]
Length (bp)	207	82	[b]
Closest relative	*T. obliquus*	*Scenedesmus acutus*	[b]
% similarity	86	82	[b]

[a] *Scenedesmus dimorphus*, now Tetradesmus dimorphus.
[b] Did not amplify.

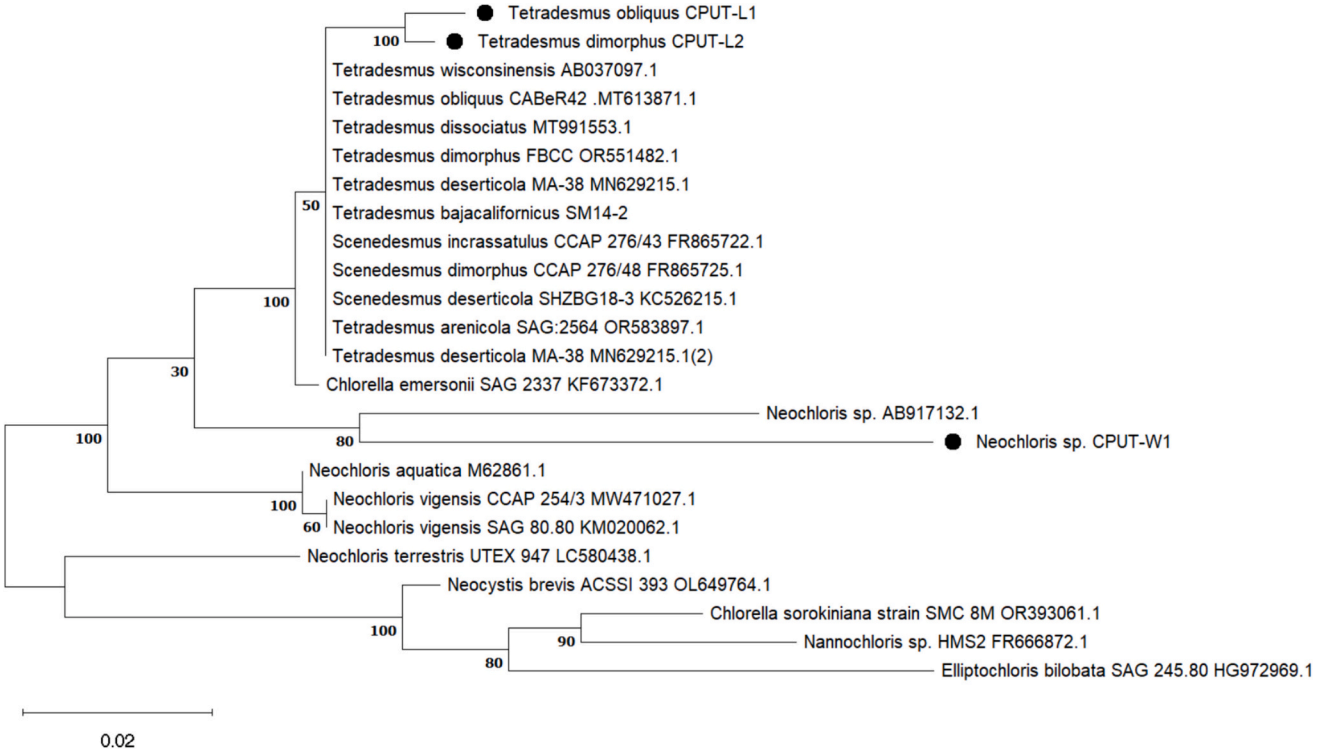

Fig. 2. D512 region Evolutionary analysis by Maximum Likelihood method. The tree with the highest log likelihood (−4238.63) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

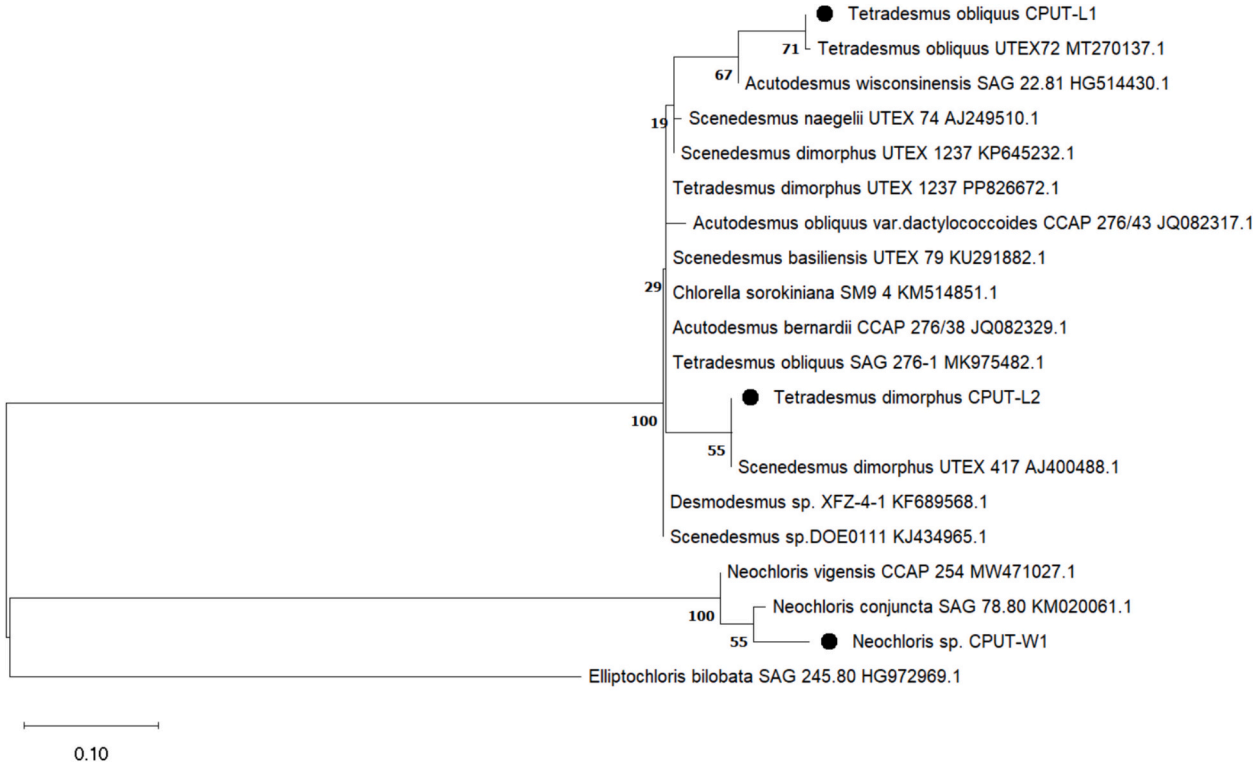

Fig. 3. ITS region Evolutionary analysis by Maximum Likelihood method. The tree with the highest log likelihood (−5974.62) is shown. The percentage of trees in which the associated taxa clustered together is shown below the branches.

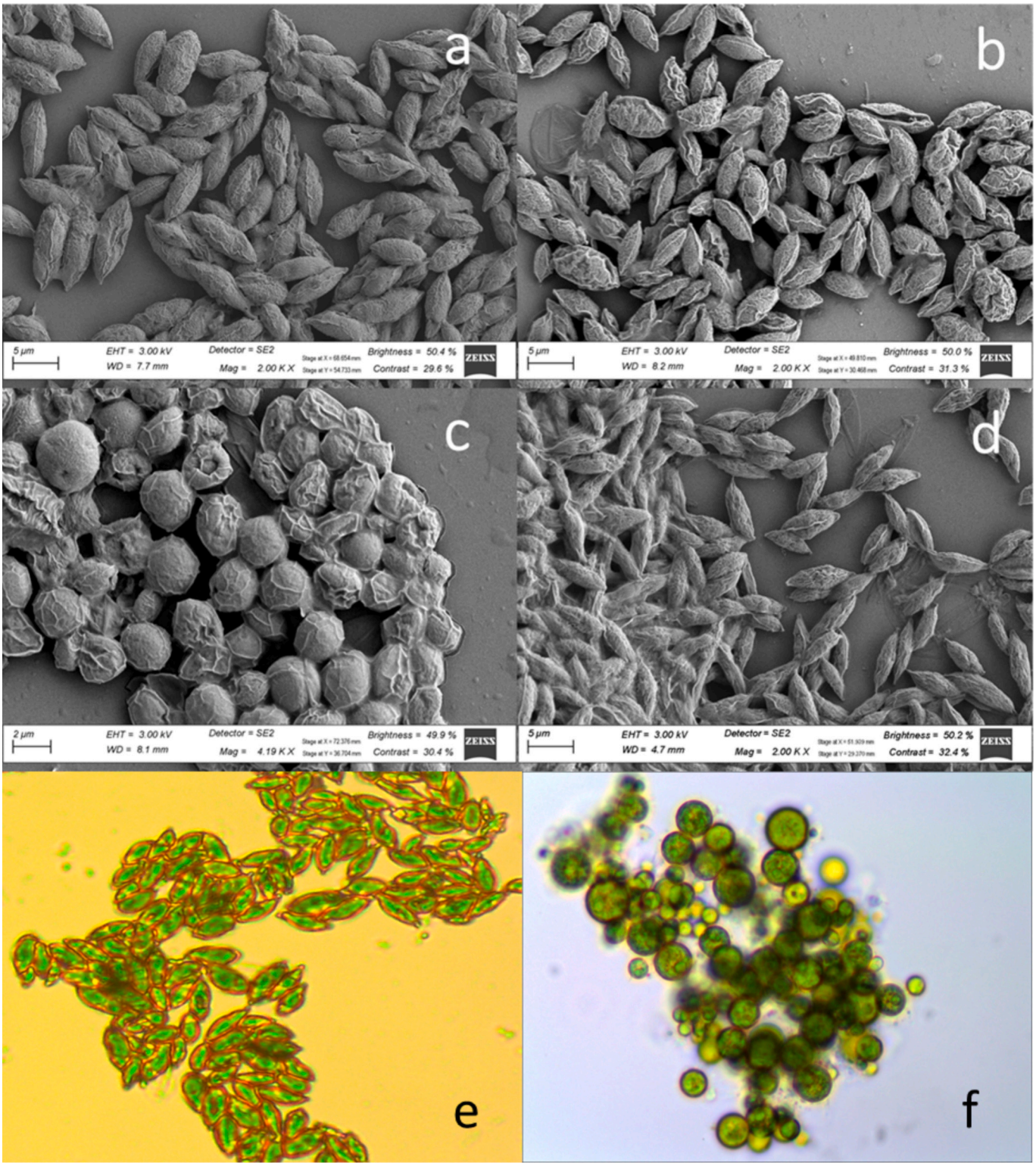

Fig. 4. Scanning electron microscopy (a–d) and light microscopy (e–f) of the microalgal strains used in the study: *Tetradesmus obliquus* CPUT-L1 (a,e), *Tetradesmus dimorphus* CPUT-L2 (b) *Neochloris* sp. CPUT-W1 (c,f) and *Tetradesmus obliquus* CCAP 276/1 A (d).

and f, respectively. Although *Tetradesmus* coenobia are classically comprised of cells arranged in groups of four or eight, the arrangement depends on the physicochemical environment [32,33,35]. Ismagulova et al. [33] found that coenobia dominated during exponential growth of *T. obliquus* IPPAS-2023 while the stationary phase was represented mostly by single cells. Some researchers have hypothesized that coenobia formation is a microalgal defense strategy [32,34]. For example, Yang et al. [34] demonstrated that chemicals released by *Daphnia carinata* induced colony formation and exopolysaccharide (EPS) production in *T. obliquus* and postulated that the EPS bound the cells together. The EPS theory of colony formation was subsequently brought into question in a study by Li et al. [35].

The morphology of *Neochloris* sp. W1 was consistent with the description by Baldisserotto et al. [36] for *Neochloris oleoabundans* strain

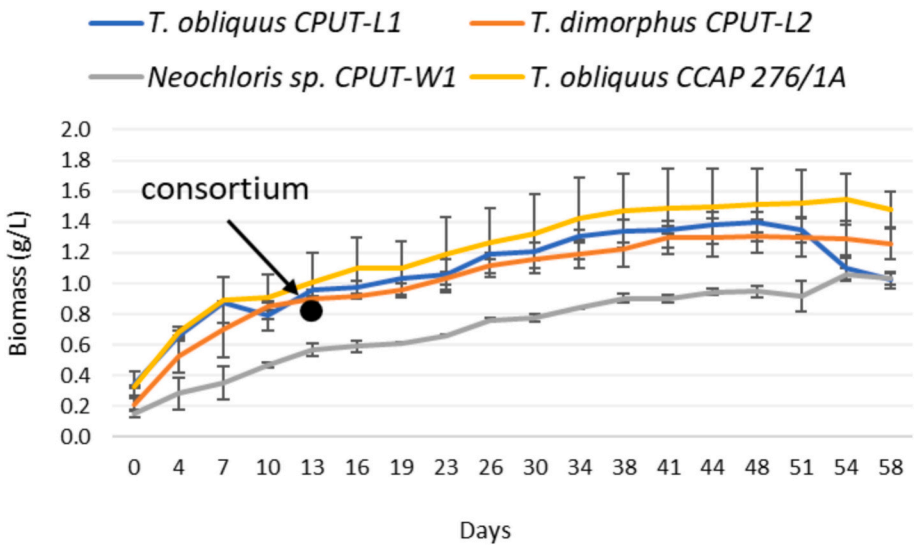

Fig. 5. Growth profiles of the individual microalgal strains and the final biomass concentration of the consortium of all four strains (represented by a dot) in treated tannery wastewater.

UTEX-1185 viz. spherical cells with approximate diameters of 2.5 μm to 3 μm with some slightly flattened cells characterized by major and minor axes of around approximately 2.5 μm to 3 μm and 2.2 μm to 2.7 μm, respectively and a large chloroplast. Organelle morphology was not considered further as major differences can be found when *N. oleoabundans* is grown under autotrophic or mixotrophic and starved or fed conditions [36].

3.2. Growth profiles of selected microalgal strains

Some microalgal strains that have consistently shown the ability to grow in TWW, most notably *Chlorella* spp. and the closely related *Scenedesmus* and *Tetradesmus/Scenedesmus* spp. produce enzymes that protect them against oxidative stress induced by toxic metals present in TWW [7]. These genera can also produce high amounts of intracellular lipids, making them ideal candidates as feedstocks for biofuel production [1,37,38]. In this study, all the strains grew in the TTWW without nutrient addition. This is the first time that growth of *Neochloris* sp. in TWW has been described.

During the experiment, the growth rates were typically highest for the first 10 days of the experiment, and the stationary phase was reached around 48 days (Fig. 5). There were no significant differences in the growth profiles obtained for *T. obliquus* CPUT-L1 *and T. dimorphus* CUT-L2 (paired *t*-TEST $p > 0.05$), but highly significant differences in the growth profiles between the other strains ($p < 0.0001$). It was not possible to obtain a temporal growth profile for the consortium because the slope of the standard graphs (Fig. 1) differed for each strain, and it was not practical to conduct temporal gravimetric biomass measurements. These results indicated that separate standard graphs need to be prepared for different microalgal strains when using OD as a proxy for biomass, and that the method is likely to be inaccurate for monitoring growth of microalgal consortia.

In terms of biomass, the highest temporal amounts measured in the individual strains (in descending order) were 1.50 ± 0.017 g/L (*T. obliquus* CCAP 276/1 A), 1.40 ± 0.07 g/L (*T. obliquus* CPUT-L1), 1.31 ± 0.11 g/L (*T. dimorphus* CPUT-L2) and 1.11 ± 0.04 g/L (*Neochloris* sp. CPUT-W1). The biomass concentration of the consortium (0.733 g/L) was within the range obtained in the individual strains (range: 0.571 g/L in *Neochloris* sp. CPUT-W1 cultures to 1.00 g/L in *T. obliquus* CCAP 276/1 A cultures). It was hypothesized that higher consortium biomass concentrations may be possible without N limitation (Section 3.3, Fig. 7).

Among other microalgae, Sehgal et al. [19] quantified the biomass of *T. obliquus* NEIST BT-A1 (0.68 g/L) and *T. dimorphus* NEIST BT1 (1.0 g/L biomass) grown in flask cultures in BG 11 medium, and both were lower than the amounts obtained with the study *Tetradesmus* strains. In 10 studies reviewed by Oliveira et al. [31] where *T. obliquus* was grown in photobioreactors, biomass concentrations of ranging from 0.56 to 20 g/L were obtained, the latter in a fed-batch system thin layer (0.5 cm) cascade system bioreactor using defined Laboratory of Algae Culture (LCA) medium [39]. In studies describing growth of *T. obliquus* in wastewater ($n = 10$), biomass yields of 0.005 g/L in municipal wastewater [40] to 3.8 g/L in poultry wastewater have been reported [31]. Biomass concentrations of 0.40 g/L and 0.54 g/L of *Neochloris pseudostigmata* UTEX 1249 and *Neochloris conjuncta* CCAP 254/1, respectively were obtained when the strains were grown in diluted synthetic centrate (40 %) in Bristol medium [41]. Lower concentrations of *N. oleoabundans* UTEX 1185 biomass (0.22 to 0.89 g/L) were obtained in artificial seawater in a flat panel photobioreactor [42].

Overall, the amount of biomass obtained in TTWW was reasonable when compared with amounts obtained with other wastewaters and synthetic media. Results are promising considering growth in TTWW obviates the need for expensive defined media or other chemical inputs.

3.2.1. Suitability of biomass for downstream applications

Table 4 contains the results obtained from characterization of the dried microalgal biomass. Interestingly, all strains showed similar compositions which often differed to the characteristics of similar strains published elsewhere. Previous studies have shown that *T. obliquus, T. dimorphus* and *Neochloris* sp. can accumulate intracellular lipids suitable in quantity and quality for biodiesel production. For example, total lipids of: (i) 23 % (wt.wt.) in *T. obliquus* NEIST BT-A1 and 25 % (wt.wt) in *T. dimorphus* NEIST BT1 grown in BG11 media ([19], (ii) 20–40 % (wt.wt.) in *T. obliquus* IPPAS S-2023 in BG 11 media (Ismagulova et al. [33]), and (iii) 29 % (wt.wt) in *N. oleoabundans* in artificial seawater in a flat panel photobioreactor under nitrogen (N) limitation [42]. As the name infers, *N. oleoabundans* can hyper-accumulate lipids, but high lipid productivity has not been described in other species of this genus. For example, only 6 % (wt.wt.) in *Neochloris conjuncta* SAG 78.80 grown in raceways in basal medium [43]. In a review of wastewater remediation studies ($n = 10$, [31]), lipid accumulation by *T. obliquus* ranged from 11 % (wt.wt.) when grown in poultry wastewater [44] to 36 % (wt.wt.) when grown in municipal wastewater [45]. Nitrogen limitation appears to promote lipid accumulation in *T. obliquus* (Ismagulova et al. 33) and

Table 4
Physicochemical characteristics of the dried biomass of the study strains grown in treated tannery wastewater ($n = 1$).

	T. obliquus CPUT-L1	*T. dimorphus* CPUT-L2	Neochloris sp. CPUT-W1	*T. obliquus* CCAP 276/1 A
Macromolecules (%wt.wt)				
Proteins	24.5	33.5	36.8	37.2
Carbohydrates (GE)	17.9	19.1	19.8	12.7
Fatty acids	10.2	13.3	7.40	9.44
Elemental CHNS (%wt.wt)				
Carbon	52.8	45.9	42.6	46.0
Hydrogen	9.61	9.11	8.44	9.22
Nitrogen	4.26	5.79	6.35	6.42
Sulfur	0.30	0.48	0.42	0.43
Major cations (g/kg)				
Sodium (Na)	8.30	7.14	7.77	4.88
Magnesium (Mg)	8.06	8.68	8.74	9.96
Phosphorus (P)	12.3	13.1	19.6	27.3
Potassium (K)	5.20	5.67	8.23	9.42
Calcium	6.46	3.78	4.89	6.63
Minor cations (g/kg)				
Aluminium (Al)	0.805	0.714	0.720	0.614
Silica (Si)	1.97	0.865	1.35	1.58
Manganese (Mn)	4.86	2.78	3.44	3.32
Iron (Fe)	1.25	0.744	1.02	0.895
Zinc (Zn)	0.176	0.0851	0.119	0.128
Minor cations (mg/kg)				
Boron (B)	30.4	38.9	41.7	24.6
Vanadium (V)	0.900	0.842	1.08	1.09
Chromium (Cr)	20.7	41.2	42.5	15.5
Cobalt (Co)	1.48	0.824	0.979	2.78
Nickel (Ni)	2.81	2.52	2.58	1.79
Copper (Cu)	11.6	8.20	6.38	9.53
Arsenic (As) (0.1)[a]	0.310	0.277	0.265	0.206
Strontium (Sr)	16.3	14.0	8.43	10.2
Molybdenum (Mo)	0.531	0.275	0.471	0.459
Cadmium (Cd) (0.05)[a]	0.047	0.055	0.044	0.083
Tin (Sn) (50)[a]	0.906	1.00	1.53	0.974
Antimony (Sb)	0.089	0.250	0.059	0.063
Barium (Ba)	31.4	17.2	19.4	23.2
Mercury (Hg) (1.2)[a]	0.029	0.018	0.014	0.014
Lead (Pb) (0.05)[a]	2.30	2.51	3.32	3.56

[a] For edible oil [48] GE = glucose equivalent; selenium was below the detection limit.

N. oleoabundans [42], as does mixotrophic growth in *N. oleoabundans* [36]. In this study, the microalgal strains accumulated relatively low amounts of lipids, despite depletion of NH_3 (Table 4). Given the low lipid concentration, the biomass generated from growth in TTWW would be unsuitable for biodiesel production.

In terms of protein, 19–56 % (wt.wt.) has previously been measured in the biomass of *T. obliquus* [31]. In *N. oleoabundans* strain UTEX-1185, the highest amount of 18 % (wt.wt) was measured in the biomass when the strain was grown under autotrophic conditions, with lower amounts when grown mixotrophically [36]. Higher protein values of 35 % (wt. wt.) were reported for *Neochloris conjuncta* SAG 78.80 grown in raceways in basal medium [43]. In other studies, the composition of *T. dimorphus* UTEX1237 grown in olive mill wastewater [46] and *Scenedesmus* sp. AMDD grown in aquaculture and agriculture effluents [47] indicated that they could be used as high protein animal feeds. Likewise, the relatively high protein concentrations of the study strains and the presence of a large range of essential trace elements suggest their suitability for aquaculture or poultry feed, provided wet-blue (chromium (Cr)) tanning is not employed. In terms of toxic metals, the concentrations of tin (Sn), mercury (Hg) and lead (Pb) fell well below the limits published jointly by the Food and Agricultural Organization (FAO) and the World Health Organization (WHO) for foods for human consumption [48]. The concentrations of arsenic (As), ranged from 0.206 mg/kg (*T. obliquus* CCAP 276/1 A) to 0.310 mg/kg (*T. obliquus* CPUT-L1), above the lower limit of 0.1 mg/kg set for edible oil, but below the upper limit of 0.35 set for husked rice. The cadmium (Cd) concentrations of *T. dimorphus* CPUT-L2 (0.055 mg/kg) and *T. obliquus* CCAP 276/1 A (0.083 mg/kg) were slightly above the limit set for a range of vegetables (0.05 mg/kg), while the other two strains were compliant. To ensure animal safety, long-term studies to assess the variability in metal content in microalgal biomass grown in TTWW would be required, followed by testing to assess the effects on growth and health of the animals [49,50].

Another option for valorisation of the microalgal biomass is co-AD with secondary TWW sludge (STWWS) because co-AD of STWWS with slaughterhouse sludge has been shown to reduce inhibition and increase biogas generation from STWWS [15,25]. Indeed, a good specific methane (CH_4) yield (200 mL/g volatile solids) was obtained from AD of digested dairy manure and silage maize as an inoculum with microalgal biomass. As a further means of beneficiation, the digestate was shown to promote growth of corn at application rates <5 t ha^{-1} [43]. Although studies such as this have shown positive results for co-AD of microalgae with various substrates (e.g. [16,43]), others have found that microalgal biomass is not always efficiently converted to CH_4 (e.g. [51,60]). It is clear from a recent review that microalgal co-AD performance is dependent on the type of co-substrate as well as the process conditions [17]. A comprehensive feasibility study co-digesting different types of

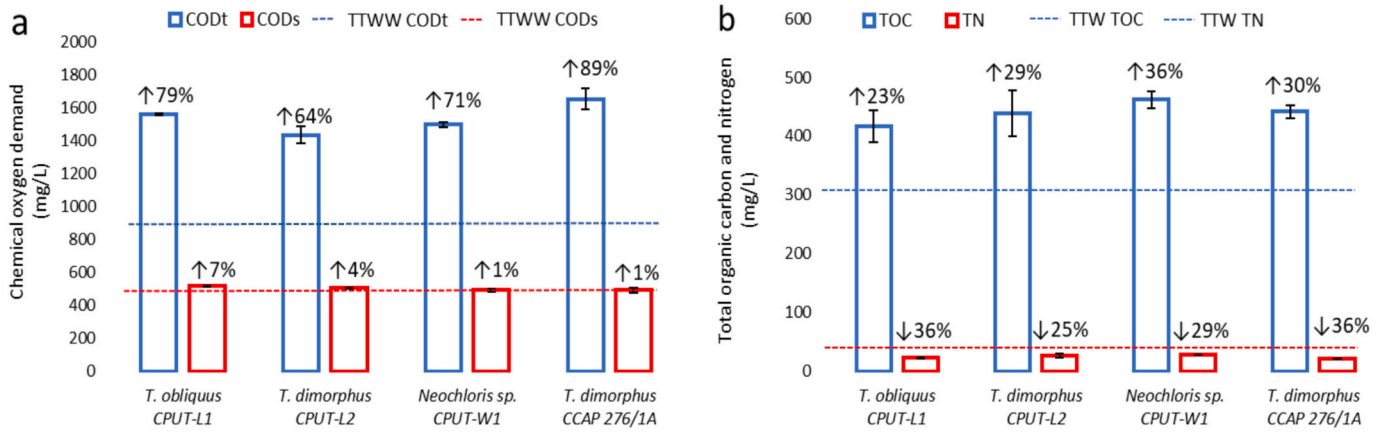

Fig. 6. Comparison of the total and soluble chemical oxygen demand (CODt, CODs) (a) and total organic carbon (TOC) and total nitrogen (TN) (b) in treated tannery wastewater at the end of the 58-day experimental period. The concentrations at the start of the experiment are indicated by the horizontal dotted lines in the graphs.

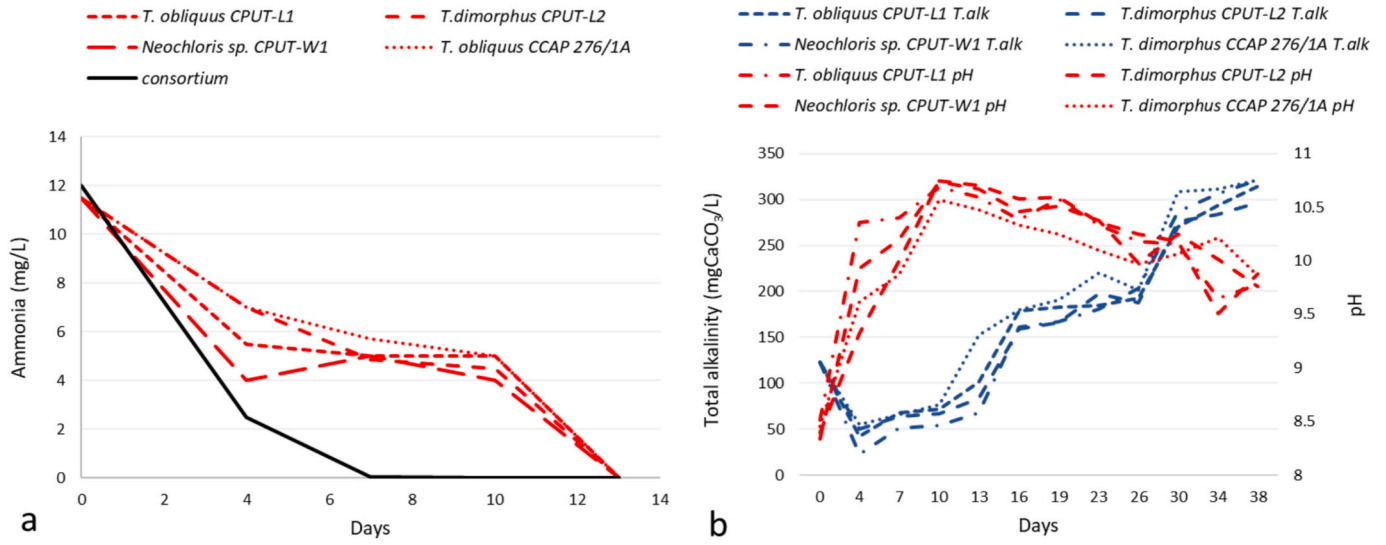

Fig. 7. Ammonia (NH_3) (a) and total alkalinity and pH (b) measurements in the microalgal monocultures.

microalgal biomass and batches of STWWS under a range of conditions is therefore recommended.

3.3. Tertiary remediation of treated tannery wastewater

Initially, it was thought that marine algae would be best suited for remediation of TTWW which is highly saline. However, the commercial marine strains that were tested (Section 2.1.1) failed to acclimate to growth with TTWW as a substrate. Of interest is that *Neochloris* strains, deemed to be freshwater microalgae, have also previously been shown to grow well in saline conditions [41,42]. In this study, *Neochloris* sp. CPUT-W1 was harvested from saline winery wastewater and was able to acclimate to growth in TWW. Similarly, Hiibel et al. [41] were able to gradually acclimate *Neochloris pseudostigmata* UTEX 1249 and *Neochloris conjuncta* CCAP 254/1 to 40 % (vol.vol) centrate/Bristol medium (average 77 mg/L Na^+, 189 mg/L Cl^-). These freshwater strains showed the highest salt tolerance of 40 microalgal strains (11 freshwater *Neochloris* strains, 11 brackish water *Nanochloropsis* strains, and 18 saltwater *Dunaliella* strains).

In terms of macronutrients, the TP concentrations in the TTWW before and after phyco-remediation were all <1 mg/L, so the results are not relevant and not included in detail in this report. The organics were measured using soluble chemical oxygen demand (CODs), total chemical oxygen demand (CODt) and total organic carbon (TOC) and the results were highly reproducible between the replicates (Fig. 6a, b). The CODs concentrations were largely unchanged, but there were 63–84 % increases in the CODt and 23–36 % increases in the TOC (Fig. 6) in the monocultures. The NH_3 was expended in the consortium culture after one week (Fig. 7). This experiment was terminated after 14 days due to visual observation that the culture was entering a period declining growth, possibly due to N limitation. At this stage, the CODt was 1675 mg/L, comparable with the *T. obliquus* CCAP 276/1 A monoculture.

The TN concentration in the TTWW was low and was reduced further during the experimental period for all the cultures (Fig. 6b). Notable increases in the influent volatile organic acid (VOA) concentration of 101 mg/L s were measured in the monoculture, from 53 % for *Neochloris* sp. CPUT-W1 to 163 % for *T. obliquus* CPUT-L1, indicating some fermentative microalgal metabolic activity. The VOA concentration in the culture containing the consortium reduced slightly to 90 mg/L.

The increase in TOC and CODt can be explained by the ability of microalgae to fix atmospheric carbon dioxide (CO_2) and replicate via autotrophic photosynthetic growth in the presence of light. The magnitude of increases will generally change under different climatic conditions because many microalgae shift towards heterotrophic growth when the light intensity is insufficient [1,2]. In high-rate algal ponds

that could potentially be used for tertiary remediation of TTWW, microalgal growth will also be influenced by the loading rates [52], the degree of shading by suspended solids, including the microalgae themselves [53], mixing [54], and the degree of mixed bacterial-microalgal growth [55]. Indeed, in the 'real world' it would be expected that significant organic utilization/removal will take place due to heterotrophic bacterial growth.

The NH_3/NH_4^+ concentrations decreased from 12 mg/L in the TTWW to below the detection limit of the test (1 mg/L) for all the strains after by 13 days, and utilization was more rapid in the consortium culture (Fig. 7a). Unlike conventional wastewater treatment systems where NH_3/NH_4^+ is removed by nitrifying bacteria that use it as a source of electrons, reducing NH_3 to nitrite (NO_2^-), microalgae take up NH_3/NH_4^+ to synthesise pigments, proteins and nucleic acids [56]. In this study, NH_3/NH_4^+ was utilized rapidly over the first few days, accompanied by increases in pH and decreases in T.Alk (Fig. 7b). As the pKa of NH_3/NH_4^+ is 9.26 at 25 °C, it was assumed that removal of NH_4^+ specifically was mainly responsible for these initial changes [56,57]. The sustained T.alk increases thereafter at higher pH values were assumed to be due to continued fixation of CO_2 and possible proton uptake as most ammonia would have been present as NH_3 at those pH levels [56,58,59].

In terms of other significant TWW pollutants, there was a slight decrease in the SO_4^{2-} concentration in the TWW, from 2 % in the cultures containing *Neochloris* sp. CPUT-W1 to 7 % in the cultures containing *T. obliquus* CPUT-L1 (TTWW = 1040 mg/L). While no HS^- was present in the TTWW, traces were measured in the final culture medium (0.23 to 1.49 mg/L in the media containing *T. obliquus* CCAP 276/1 A and *T. obliquus* CPUT-L1, respectively). Removal rates of chloride ions (Cl^-), ranged from 8 % (*T. obliquus* CCAP 276/1 A) to 19 % (*T. obliquus* CPUT-L1). Given the high concentrations of Cl^- in the TTWW (1975 mg/L), this could still be notable in terms of bioremediation.

4. Conclusions

In summary, the results obtained during the flask mono-culture studies indicated that TTWW can be successfully used 'as is' as a growth medium for culture of selected strains of microalgae, removal of residual NH_3, and reduction of Cl^- in TTWW. Separation of the biomass from the TTWW is imperative to ensure that the CODt of the final effluent does not increase. Fortunately, the study strains settled rapidly in the TTWW, probably due to the formation of large coenobia which may be the result of chemical stressors present in the TTWW. Gravity separation of microalgae from wastewater is simple and cost effective, adding to the potential feasibility of the process at scale for production of utilizable biomass in a TWW biorefinery. The chemical composition of the individual strains showed low variability. This suggests that the biomass of a microalgal consortium may not vary significantly due to species succession that is expected in a full-scale phyco-remediation system. This is a promising sign when considering downstream valorisation options such as utilization of the biomass as an animal feed supplement.

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CRediT authorship contribution statement

A. Ranjan: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **M. Lilly:** Writing – review & editing, Writing – original draft, Validation, Methodology. **N. Deepnarain:** Validation, Data curation. **P. J. Welz:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Declaration of competing interest

The authors have no relevant financial or non-financial competing interests to declare.

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Data availability

Data will be made available on request.

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