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Screening native isolates of cyanobacteria and a green alga for integrated wastewater treatment, biomass accumulation and neutral lipid production

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ABSTRACT

The value and efficiency of microalgal biofuel production can be improved in an integrated system using waste streams as feed-stock, with fuel-rich biomass and treated wastewater being key end-products. We have evaluated seven native cyanobacterial isolates and one native green alga for their nutrient removal, biomass accumulation and lipid production capacities. All native isolates were successfully grown on synthetic wastewater mimicking secondary treated municipal wastewater (without organic carbon). Complete phosphate removal was achieved by the native green alga, isolated from Tvärminne (SW Finland). Optimisation of the C:N ratio available to this strain was achieved by addition of 3% CO_2 and resulted in complete ammonium removal in synthetic wastewater. The native green alga demonstrated similar nutrient removal rates and even stronger growth in screened municipal wastewater, which had double the ammonium concentration of the synthetic media and also contained organic carbon. Sequencing of the genes coding for 18S small rRNA subunit and the ITS1 spacer region of this alga placed it in the Scenedesmaceae family. The lipid content of native isolates was evaluated using BODIPY (505/515) staining combined with high-throughput flow cytometry, where the native green alga demonstrated significantly greater neutral lipid accumulation than the cyanobacteria under the conditions studied.

1. Introduction

In order to preserve our current way of life, humans are now faced with the challenge of limiting the impact of our progress on the natural environment that supports us. This is important both in the treatment of waste products, such as contaminated water and waste CO_2 gases; and in the production of energy, particularly that which can be stored as fuel. In Finland, the importance of these issues is reflected in international agreements, including the Helsinki Convention on the Protection of the Marine Environment of the Baltic Sea Area (HELCOM), and Finland's national action plan for promoting energy from renewable sources (pursuant to 2009/28/EC). Both agreements set ambitious targets. HELCOM recommendation 28E/5 [1] stipulates 10% stricter wastewater treatment standards than the Urban Waste Water Treatment Directive 91/271/EEC [2], in setting wastewater phosphorus removal targets of 90% (population equivalents >10,000), whilst Finland's renewable action plan sets a target of 38% of (gross final consumption) energy to be supplied by renewable sources by 2020 [3]. Increasingly efficient conversion of solar energy to stored fuel is integral to meeting such targets.

Green algae and cyanobacteria are distinct photosynthetic organisms able to rapidly convert solar energy to carbon-based compounds. They are attractive raw materials for biofuel production due to their ability to capture CO_2 and the small impact that cultivation has on agriculture and land availability. Importantly, the economic value, energy, and resource efficiency of photosynthetic biofuels can be considerably improved when employing waste streams as feed-stocks [4,5]. Nutrient resource reuse is particularly important in the face of diminishing phosphorus reserves and threats to global food security (see, for example, [6]). Despite algal wastewater treatment being promoted as long ago as the 1950s [7], it has yet to be adopted as a conventional approach [4]. The phototrophic nature of these organisms presents both new opportunities and new challenges to standard process design used in conventional wastewater treatment. This, and the subsequent variety of strains and experimental conditions investigated thus far, have yielded a variety of nutrient removal efficiencies (see [8] for a brief summary), presenting large scope for optimisation.

Neutral lipids are accumulated as triacylglycerols (TAGs) in algae when the organism is under stress or other adverse environmental

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conditions, with levels of up to 50% of lipids per dry weight having been observed [9]. This tendency of algae to accumulate TAG has ensured a greater focus on their potential as a raw material for biodiesel production than cyanobacteria, which typically store carbon as glycogen and/or polyhydroxyalkanoates [10]. However, cyanobacteria do contain lipids available for direct conversion to biodiesel [11,12] and have previously been touted as suitable for high-rate lipid-production due to their accumulation of lipids in thylakoid membranes, their high levels of photosynthesis and rapid growth rate [13].

Whilst it is commonly stated that TAGs are not found in cyanobacteria, the converse has been sporadically reported since at least 1993 (see [14] and references therein). Moreover, TAGs found to accumulate under conditions of nutrient stress have recently been detected in lipid droplets of the cyanobacterium *Nostoc punctiforme* [15] and increases in lipid content have also been reported in nutrient starved cyanobacteria originating from Sweden [16]. However, the most well recognised advantages of cyanobacteria are the relative ease of their genetic manipulation and their ability to secrete fatty acids and other carbon-based products into their surrounding environment (see, for example: [10,17,18]). Thus, the identification and characterisation of unique isolates of both algae and cyanobacteria remains an important step in realising phototrophic bioenergy production.

The University of Helsinki Culture Collection (UHCC), containing more than 1000 isolates from the unique Finnish environment, presents great opportunity for exploring the biofuel potential of these native organisms. Employment of isolates native to a particular area may be advantageous in their inherent suitability to environmental conditions; a potential benefit in developing an energetically efficient biofuel production platform. We have already studied the biohydrogen photoproduction of isolates from the UHCC [19], and the extension and stabilization of production through improved light conversion efficiency of cells entrapped in thin alginate films [20,21]. We expect that native isolates may have an advantage at lower bioreactor temperatures and under lower light conditions. The current work investigates the potential for minimising the energy and fertiliser requirements of growing cyanobacteria and algae through the employment of an integrated system which simultaneously treats wastewater and generates biomass for use in biofuel (bio-hydrogen or biodiesel) production.

2. Materials and methods

2.1. Strain selection and growth

Native isolates were obtained from the University of Helsinki Culture Collection (UHCC) and were maintained, along with model cyanobacteria strain *Synechocystis* PCC 6803, herein *Synechocystis*, and control alga *Chlorella vulgaris* (UTEX 265), herein *Chlorella*, in standard BG11 medium (buffered with 5 mM HEPES/NaOH, pH 7.4) under continuous low light (approximately 15 μmol photons m^{-2} s^{-1} photosynthetic active radiation (PAR)) at room temperature. For all experiments, cell cultures underwent an antecedent controlled growth period under exact experimental conditions. This was to allow poly-phosphate stores to reach levels that may be expected in a semi-continuous growth system. For experiments performed in municipal wastewater (mWW), antecedent growth was undertaken in synthetic media, with NH_4^+-N and PO_4^{3-}-P concentrations adjusted to match those of the mWW.

A guide to the UHCC identification code, taxonomy and place of isolation are provided for the native cyanobacteria and alga employed in this study (Table 1). To demonstrate the diversity of native cyanobacterial strains, a neighbour-joining tree was generated from the alignment of the available 16S DNA sequences [22,23] using MEGA 6 software (http://www.megasoftware.net/) with bootstrapping at 1000 replicates (Fig. S1). Included for reference are the 3 closest relatives of each native isolate (found using BLAST searches and excluding uncultured hits), *Synechocystis* sp. PCC 6803 (used as a control strain in this study) and the species employed in the study of Capella-Gutierrez et al. [24] to represent the four major phylogenetic groups of Cyanobacteria.

2.2. Screening for biomass accumulation and nutrient removal

Biomass and nutrient removal screening was performed in synthetic wastewater media (synWW), which was based on BG11 media with nitrogen and phosphorus concentrations adjusted to those of Shi et al. [25]. Concentrations of nitrate-N (NO_3^--N) as $NaNO_3$, ammonium-N (NH_4^+-N) as NH_4Cl, and phosphate-P (PO_4^{3-}-P) as K_2HPO_4 were approximately 3, 21, and 4 mg/L respectively. The pH of the media was adjusted to 7.4, buffered with 5 mM HEPES/NaOH, and autoclaved prior to use. Growth was performed in 500 mL batch culture from a low starting concentration ($OD_{750} = 0.1$) and under low energy conditions (40 μmol photons m^{-2} s^{-1} continuous light, 22 °C and mixing at 100 rpm) for 14 days. Conditions were chosen to target isolates best suited for generating biomass in synWW, without a large energy input. All isolates evaluated were unicellular to allow easy comparison of biomass accumulation using optical density (OD) at 750 nm, OD_{750}.

Samples were analysed approximately daily for: OD_{750}; Chlorophyll *a* (Chl *a*) (according to Meeks et al. [26]); NH_4^+-N (using Merck kit 100683, according to the phenate method); and PO_4^{3-}-P (using Merck kit 114848, according to the ascorbic acid method).

All experiments included a method blank, which consisted of the sample matrix but did not have cyanobacteria or algae added. At each time point, OD_{750} and Chl *a* were adjusted to account for any contribution from the method blank (sample − blank). In synWW, values of OD_{750} and Chl *a* from the method blank were negligible (i.e. no contaminating growth was observed). Nutrient removal was calculated at each time point relative to the blank control (at the same time point) using the formula below:

$$\%\,\text{removal}(t) = 100 * \left(([b]_t - [s]_t)/[b]_t\right) \quad (1)$$

where t is time, b is the method blank, and s is the sample.

Table 1
Native cyanobacteria and alga employed in this study.

UHCC identifier	Place and year of isolation	Taxonomy	Reference
1TU21S5	Tuusulanjärvi (2001)	*Synechococcus* sp.	Rajaniemi-Wacklin et al. [23]
1TU44S8	Tuusulanjärvi (2001)	Unknown	N/A
0TU37S4	Tuusulanjärvi (2000)	*Snowella litoralis*	Rajaniemi-Wacklin et al. [22]
SYKE695	Unknown	*Microcystis* sp.	Personal communication, Lyudmila Saari
UHCC0027	Tvärminne (2010)	*Scenedesmus* sp.	This study
1TU39S1	Tuusulanjärvi (2001)	*Synechococcus* sp.	Rajaniemi-Wacklin et al. [23]
0TU24S4	Tuusulanjärvi (2000)	*Synechococcus* sp.	Personal communication, Anne Ylinen
SYKE2088A	Unknown	*Microcystis* sp.	Personal communication, Lyudmila Saari

2.3. Screening for high light and high CO_2 tolerance

To evaluate high light tolerance, cells were grown in a dilution series with starting OD_{750} values of 0.01, 0.05, 0.1, 0.2, 0.4, and 0.6. Experiments were performed in 24-well culture plates for four days under 500 μmol photons m^{-2} s^{-1} of overhead continuous light (atmospheric CO_2, 22 °C, mixing at 100 rpm). After four days, OD_{750} and Chl *a* were determined as described above.

For high CO_2 experiments, cells were grown from a starting OD_{750} of 0.1, in 24-well culture plates for four days under 40 μmol photons m^{-2} s^{-1} of continuous light (22 °C, mixing at 100 rpm) in the presence of 3% CO_2. After four days, OD_{750} and Chl *a* were determined as described above.

2.4. Optimising conditions for enhanced UHCC0027 performance

Following the demonstrated tolerance of UHCC0027 cells to both high light and high CO_2 conditions on micro-scale, a larger scale evaluation was undertaken to address possible nutrient and light deprivation conditions of the initial biomass generation and nutrient removal screening experiment. Biomass generation and nutrient removal were investigated specifically for the most promising native isolate, UHCC0027, in both synWW (with 25 mM HEPES) and screened municipal wastewater (mWW).

Experiments were undertaken as described for the screening experiment, except that they were performed under higher light intensity (220 μmol photons m^{-2} s^{-1}) using a 16:8 h light:dark photoperiod (experiments performed in synWW were additionally evaluated under continuous light conditions). To investigate the effect of CO_2, experiments were performed under both atmospheric (approximately 0.04%), and 3% CO_2. Experiments performed under atmospheric CO_2 ran for 10 days, those at 3% CO_2 ran for 5 days. Screened municipal wastewater (mWW) from the Varissuo district (Turku, Finland) was supplied by Clewer Technology Oy and contained average values of 42 mg/L NH_4^+-N and 5 mg/L PO_4^{3-}-P.

2.5. Monitoring photosynthetic performance

The photosynthetic performance of cells was monitored for the high carbon (3% CO_2) experiments in both synWW and mWW using DUAL-PAM 100 (Walz, Effelrich, Germany). Cells were harvested and adjusted to a final Chl *a* concentration of 5 μg/mL in fresh synWW media. The steady-state Chl *a* fluorescence level (Ft) was determined after illumination of the cells with actinic red light intensity of ~126 μmol photons m^{-2} s^{-1}. The maximum fluorescence level at light (Fm′) was obtained by firing a saturating light pulse (300 ms/3000 μmol photons m^{-2} s^{-1}) to illuminated cells. The effective Photosystem II yield, Y(II), was calculated by: Fm′ − Ft / Fm′. Light response curves of Y(II) were recorded during the step-wise increase of light intensity form 0 to 829 μmol photons m^{-2} s^{-1}, with a 30 s illumination at each step.

2.6. Screening for neutral lipid content

Cells were grown for 1 week in synWW from OD_{750} 0.2 in 24-well culture plates at 50 μmol photons m^{-2} s^{-1} of overhead continuous light (22 °C, mixing at 100 rpm). After 1 week, the OD_{750} was determined and cells diluted to a final OD_{750} 0.25. BODIPY (4,4-difluoro-1,3,5,7,-tetramethyl-4-bora-3a,4a-diaza-s-indacene) (505/515) lipophillic dye (stored in DMSO according to [27]) was applied to cells at a final concentration of 1 μg/mL (0.1% DMSO) and incubated for 1.5 min before flow cytometry analysis. Samples were run on a Becton Dickson LSRII flow cytometer equipped with a 488 nm excitation laser. The BODIPY fluorescence signal was collected using a 530/30 nm band-pass filter and data collected using FACSDiva software v. 6.1.3 prior to analysis using Flowing software v.2.5 (http://www.flowingsoftware.com/). The gating strategy employed was based on that of [28] using fluorescence in the PE-Cy7 channel (780/60 nm band-pass filter) versus Chl autofluorescence in the PerCP-Cy5-5 channel (695/40 nm band-pass filter) to remove non-cell signals from the population of interest. Unstained cell samples and a cell free sample (to which BODIPY was added) were used as controls. Analysis was performed in triplicate from biological replicates.

The total lipid content of cells was determined according to Ryckebosh et al. [29]. Briefly, approximately 10 mg of lyophilized biomass was vortexed in glass tubes with 400 μL methanol for 30 s. Chloroform (200 μL) and MQ-water (40 μL) were added and vortexing was repeated. Finally, chloroform (200 μL) and MQ-water (200 μL) were added and vortexed for 30 s before centrifugation (2000 rpm, 10 min). The oil layer was collected and passed through a column of anhydrous sodium sulfate to absorb residual moisture. Solvent was removed at 40 °C under nitrogen. The pellet was again extracted with chloroform:methanol 1:1 (400 μL) and MQ-water (120 μL). Gravimetric yields from both extractions were summed to determine the total lipid content.

2.7. Confocal microscopy

Cells were stained using BODIPY as described previously and deposited on 1% agarose pads for imaging on a Zeiss LSM510 META Confocal Laser Scanning Microscope (CLSM). Images were scanned using a 488 nm Argon laser and collected using long-pass filter 650 nm for Chl auto-fluorescence and 500–550 nm band-pass filter for BODIPY detection. Images were captured using Zeiss LSM5 software and ImageJ v.1.48 (http://imagej.nih.gov/ij/index.html) was used to adjust brightness and contrast, to crop, colour and label images, merge images, and to convert images to .tif format.

2.8. Identification of strain UHCC0027

UHCC0027 cells were lysed by grinding in liquid nitrogen and incubating in SDS buffer (200 mM Tris–HCl pH 7.5, 25 mM NaCl, 25 mM EDTA pH 8.0, 0.5% SDS). DNA was extracted twice using phenol: chloroform:isoamylalcohol (24:24:1), once using chloroform only, and precipitated using ethanol. PCR was performed using primers specific to the 18S small subunit ribosomal DNA and to the Internal Transcribed Spacer 1 (ITS1) region. All PCR reactions were run in 40 μL volumes containing 4 μL 10× Taq buffer, 1 μL Taq polymerase, 1 μL dNTPs, 0.5 μM of each primer, and 2 μL of template DNA. PCR amplification was performed using an MJ Research PTC 200 Thermal Cycler with an initial 5 min denaturation at 95 °C, followed by 30 cycles of 95 °C for 45 s, 52 °C for 45 s, 72 °C for 2 min, and a final extension of 72 °C for 15 min.

PCR primer pair SSU1 (5′-TGGTTGATCCTGCCAGTAG) and SSU2 (5′-TGATCCTTCCGCAGGTTCAC) [30] were initially used for 18S rRNA gene sequencing. However, as gene coverage was insufficient, primers SSU1_27 (5′-GCATGTCTAAGTATAAACTGC) and SSU2_27 (5′-TCAATTCCTTTAAGTTTCAGC), were designed using Bioedit v.7.2.5 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), based on homologues of the initial sequence data. Primers ITS1F_27 (5′-GCTGAAACTTAAAGGAATTGA) and ITS1R_27 (5′-AGCCAAGATATCCGTTGTTG) were similarly designed to amplify the ITS1 region of strain UHCC0027.

All sequencing were performed at the Turku Centre for Biotechnology (Finland) using primers detailed above and the ABI PRISM BigDye® Terminator v3.1 Ready Reaction Cycle Sequencing Kit. A Neighbour-Joining tree was generated from the alignment of 18S and ITS1 DNA sequences of the indicated algal strains using MEGA 6 software (http://www.megasoftware.net/) with bootstrapping at 1000 replicates. Algae included in the phylogenetic tree were chosen based on the closest NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) matches containing 100% query coverage of the 18S & ITS1 regions. *C. vulgaris* was included for reference.

3. Results and discussion

3.1. Screening for biomass accumulation and nutrient removal in synthetic wastewater

Under low energy conditions (22 °C, 40 μmol photons m^{-2} s^{-1} and no bubbling) cell growth generally followed a standard microbial curve, with all cyanobacteria except *Synechocystis* and 1TU44S8 experiencing a lag phase of approximately 4 days. The native green alga UHCC0027 and the control strain *Chlorella* also lacked strong lag phases. Cyanobacteria and algae took a minimum of 8 days to reach stationary phase, but this was at lower OD_{750} and longer residence time than would be useful for either biofuel generation or wastewater treatment (Fig. 1A). The native green alga UHCC0027 demonstrated the most rapid growth, reaching a maximum OD_{750} at 12 days and decreasing thereafter. Two of the native cyanobacterial isolates, 1TU44S8 and 1TU21S5, demonstrated increases in OD_{750} that were approximately equal to both control strains, *Synechocystis* and *Chlorella*.

Ammonium removal reached maximum levels of 50%, with the lowest final concentration being 10.5 mg/L (Fig. 1b), falling short of removal required to meet EU standards for release to sensitive waters subject to eutrophication [2]. None of the isolates stood out as being particularly efficient at removing ammonium and, since none were nitrogen fixing, this was likely due to either light limitation or nutritional imbalance. The N:P molar ratio of synWW used in this test (13:1) was just below that of the Redfield ratio (16:1) [31], indicating that of the two nutrients, nitrogen would be expected to be limiting in our experiments. However, deviations from the 'ideal' Redfield ratio of phytoplankton are commonly observed [32] and the low levels of NH_4^+-N uptake clearly indicate that it was not limiting in our system. Thus, of the major nutrients required for growth, it was apparent that additional carbon was required for enhanced biomass accumulation.

The removal of phosphorus from wastewater is important for protecting sensitive water catchments, and the opportunity to recover this valuable nutrient could be an additional benefit of algal and/or cyanobacterial wastewater treatment. Both organisms have the ability to perform luxury uptake of phosphorus (uptake beyond nutritional requirements), by concentrating phosphorus in poly-phosphate stores [33]. Cellular phosphorus can be recovered through anaerobic digestion (AD) of spent biomass [34] with the production of methane fuel an additional benefit of the AD process. Phosphate removal in synWW media was clearly better than ammonium removal for most isolates, with UHCC0027 achieving complete removal by the end of the 14 day study (Fig. 1D). The affinity for phosphate uptake shown by UHCC0027 is most obvious when plotted relative to rates of biomass and Chl *a* accumulation, whereby the relative rate of PO_4^{3-}-P removal is approximately 3.3 times that of NH_4^+-N (Fig. S1). As a comparison, *Chlorella* removal rates relative to OD_{750} and Chl *a* were almost equal for both nutrient types.

Wide varieties of nutrient removal rates are reported for both green algae and cyanobacteria, over a number of different conditions. A good example of this can be found in the total nitrogen (TN) and total phosphorus (TP) removal rates reported in the summary of Cai et al. [8], which range from 8 to 100% for both TN and TP removal

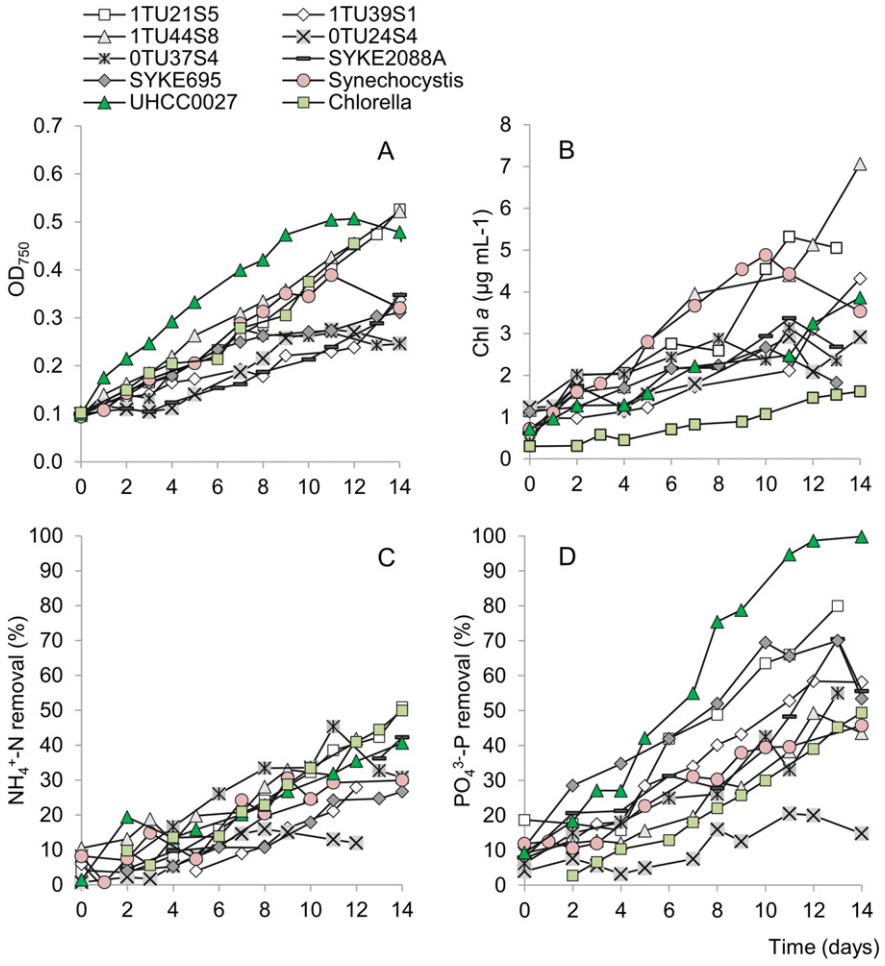

Fig. 1. Screening of cyanobacteria and algae for: biomass accumulation, as OD_{750} (A) and Chl *a* (μg mL^{-1}) (B); and nutrient removal, as NH_4^+-N removal (%) (C) and PO_4^{3-}-P removal (%) (D). UHCC0027 (dark green triangles), *Chlorella* (light green squares) and *Synechocystis* (light red circles) are highlighted.

by green algae and 84 to 100% for TN and 72 to 100% for TP removal by cyanobacteria. At first glance, the numbers appear to support cyanobacteria as the better candidates for nutrient removal, but the reported range considers only 3 studies, compared to 13 studies of green algae. Whilst this is a relatively small sample of the data currently available in the literature, it clearly demonstrates the results of a multitude of conditions applied in different laboratory and field trials (including wastewater composition, initial cell population densities, reactor temperature, pH, light irradiance and photoperiod, residence time, and mode of operation). Thus, direct comparisons to removal efficiencies between studies are troublesome. It is however, important to assess nutrient removal performance in regard to discharge standards. The NH_4^+-N removal rates demonstrated in this screening were insufficient to meet these standards, and whilst PO_4^{3-}-P removal by strain UHCC0027 was impressive, the hydraulic residence time (HRT) required was too long to be practicable on a wastewater treatment plant. Thus the optimisation of conditions was required.

3.2. Screening for high light and 3% CO_2 tolerance

In order to optimise nutrient removal and biomass accumulation rates, we undertook a micro-scale screening for cell response to increases in both light and CO_2, either or both of which may have been limiting in our initial experiments.

The tolerance of cyanobacterial and algal cells to 500 μmol photons m^{-2} s^{-1} high light was determined on micro-scale using a dilution series experiment, where strain UHCC0027 demonstrated superior growth over the other isolates. This was most evident at the lowest starting dilution (OD_{750} = 0.01), where it achieved growth of approximately 0.45 Absorbance (Abs) units greater than *Synechocystis* by the final day of the experiment (Fig. 2A). At higher starting levels of OD_{750}, the final growth advantage that UHCC0027 had over the other alga and cyanobacteria was minimised such that, from the starting OD_{750} of 0.4, there was no significant difference in final OD_{750} values demonstrated by *Synechocystis*, 0TU24S4, and UHCC0027 cells (Fig. 2a).

UHCC0027 also demonstrated the best response to increased CO_2 levels, where 4 days of growth from an OD_{750} of 0.1 resulted in a final OD_{750} of 1.55 (Fig. 2B). This is an increase of 0.65 Abs units over the final OD_{750} obtained in the high light experiment (final OD_{750} = 0.9, Fig. 2A), indicating the potential employment of increased CO_2 for improved biomass yield. Moreover, efficient CO_2 sequestration is an additional benefit to the integrated wastewater treatment and biomass production platform we are aiming to achieve, with the introduction of CO_2 via waste flue gas the most attractive option. However, since flue gas contains CO_2 at levels of approximately 25%, along with other noxious gases such as SO_x and NO_x [35]; tolerance would need to be further investigated.

Cyanobacterium SYKE 695 performed poorly under both increased light and increased CO_2 conditions and was the only strain that did not demonstrate an increase in biomass under the latter condition. Under the increased CO_2 condition, both algae demonstrated comparatively higher Chl *a* levels than those demonstrated by cyanobacteria (Fig. 2B). This is the reverse of the trend observed in the nutrient removal screening experiment, performed under atmospheric CO_2 levels, where cyanobacteria demonstrated higher Chl *a* (Fig. 1C) than the algae. Despite this, the Chl *a* values relative to OD_{750} were actually quite consistent between the two CO_2 conditions for algae, whilst the cyanobacteria demonstrated a decrease of Chl *a* relative to OD_{750} under high carbon conditions.

3.3. Optimising conditions for enhanced UHCC0027 performance

A deeper investigation into optimising biomass generation and nutrient removal rates was undertaken for the most promising isolate, UHCC0027, by performing 500 mL batch mode experiments at a higher light intensity of 220 μmol photons m^{-2} s^{-1}, using a 16:8 h light:dark photoperiod, and under either atmospheric (approximately 0.04%), or 3% CO_2 (Fig. 3) in both synWW and mWW.

3.3.1. Effect of higher light intensity

Comparing results obtained in synWW (Fig. 3) to those of the screening experiment performed at 40 μmol photons m^{-2} s^{-1} (Fig. 1), it appears that increasing the light intensity under atmospheric CO_2 conditions did not improve biomass accumulation or Chl *a*. However, NH_4^+-N removal did increase approximately 10% under the high light condition (Day 10, Figs. 1C and 3C). Nitrogen is required for protein synthesis, for photoautotrophic growth, this is specifically important for the maintenance of the many proteins associated with the photosynthetic apparatus [36]. Thus, the observed increase in ammonium uptake could be due to a higher demand on nitrogen under high light, required to maintain photosynthetic pigment protein complexes under efficient photo-damage/repair cycles [37].

It may be expected that increased light intensity would also increase the rate of phosphorus uptake, due to an increased energy yield (ATP) linked to a higher photosynthetic rate. Such an effect has previously been demonstrated by Powell et al. [38], whereby a higher amount of acid-soluble polyphosphate was accumulated by a Scenedesmus-dominated microalgal community grown under 150, as opposed to 60 μmol photons m^{-2} s^{-1}. However, in our study, both OD_{750} and

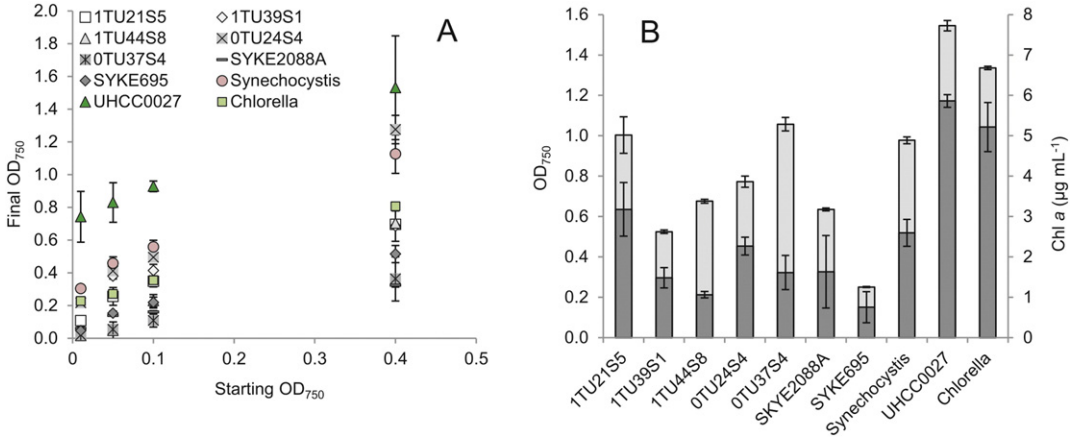

Fig. 2. Screening for growth response to 500 μmol photons m^{-2} s^{-1} high light (A) and to 3% CO_2 (B). High light data (A) is Final OD_{750} (y axis) as a function of Starting OD_{750} (x axis). UHCC0027 (dark green triangles), *Chlorella* (light green squares) and *Synechocystis* (light red circles) are highlighted. CO_2 data (B) is presented as OD_{750} (left y axis) represented by columns in light grey (rear) and Chl *a* (μg mL^{-1}, right y axis) represented by columns in dark grey (front), after four days of growth.

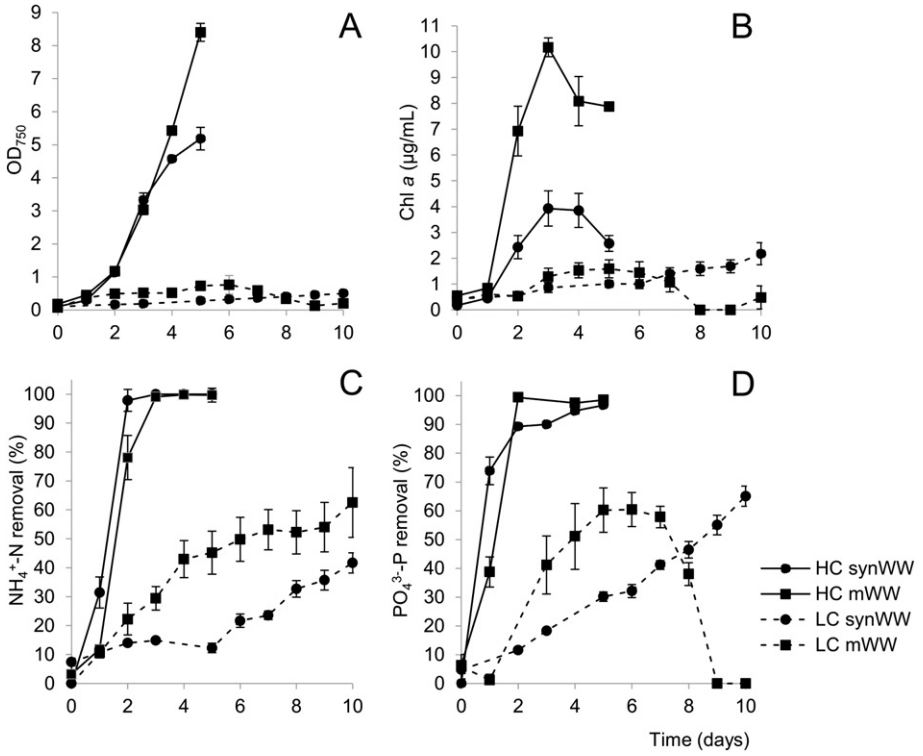

Fig. 3. Effects of high light intensity and CO_2 amendment on UHCC0027 biomass accumulation, as OD_{750} (A) and Chl *a* (μg mL^{-1}) (B); and nutrient removal, as NH_4^+-N removal (%) (C) and PO_4^{3-}-P removal (%) (D). HC indicates high (3%) CO_2 (unbroken lines) and LC indicates low (atmospheric) levels of CO_2 (dashed lines). Experiments were performed in either synthetic wastewater media (synWW, circle symbols, initial composition approximately 21 mg/L NH_4^+-N and 4 mg/L PO_4^{3-}P), or screened municipal wastewater (mWW, square symbols, initial composition approximately 42 mg/L NH_4^+-N and 5 mg/L PO_4^{3-}P).

Chl *a* were not improved by increased light intensity (day 10, Figs. 1A, B, 3A and B), and thus increased consumption of phosphate was not observed.

3.3.2. Effect of carbon availability

Contrasting the small effect that increased light intensity appeared to impart, the benefit of performing experiments under 3% CO_2 was evident for all parameters measured, indicating that carbon limitation may have been the greatest constraint to biomass accumulation and nutrient removal in the initial screening experiment. Indeed, increased CO_2 (and importantly, increased C:N ratio available to cells) resulted in the exponential accumulation of synWW biomass after only 1 day, and, within only 2 days, almost 100% and 90% removal was achieved for NH_4^+-N and PO_4^{3-}-P respectively (Fig. 3).

However, since experimental conditions were nitrogen sufficient, UHCC0027 cells would not have accumulated large carbohydrate reserves, meaning that limiting either light or CO_2 (effectively limiting photosynthate) would have constrained nitrogen assimilation in the screening experiment ([39] and references therein). Thus, it is difficult to completely separate the effect of increased light intensity from the improvements observed under the higher carbon condition.

3.3.3. Effect of photoperiod

Along with the 16:8 light:dark regime, experiments performed in synWW were also performed under continuous light. This was to determine whether there would be any biomass or nutrient removal penalty for operation in day/night mode. As there was no significant difference observed in performance between the two different light regimes, power savings could be made by operating in day/night mode (Fig. S3). The 16:8 light:dark photoperiod employed in this study fits well with the outcomes of Bouterfas et al. [40], who recommended a 12 to 15 hour illumination in considering the balance between the cost of energy and corresponding algal biomass production in batch culture.

3.3.4. Municipal wastewater as a growth medium

Experiments performed in screened municipal wastewater (mWW) demonstrated similar responses to light and CO_2 amendment as those performed in synWW. However, growth in mWW, containing approximately double the ammonium present in synWW, improved the performance of UHCC0027 cells for almost all parameters investigated (Fig. 3). Under 3% CO_2 conditions, both the biomass and Chl *a* amount produced by UHCC0027 in mWW were significantly greater than was produced in synWW. The early difference in Chl *a* was likely due to the higher nitrogen availability of the mWW, which also ensured a high photosynthetic yield under the higher CO_2 condition (Fig. 4). An exponential increase in OD_{750} was observed shortly after that of Chl *a*. Shading effects in the relatively highly coloured mWW (containing colloidal and settable solids, including indigenous bacteria) may also have induced a high rate of Chl *a* biosynthesis. Although, the small difference in early Chl *a* levels between the two media under atmospheric CO_2 levels suggests that this was not a major factor.

Whilst early nutrient removal rates in mWW under 3% CO_2 did not demonstrate a clear advantage over those in synWW, it should be noted that, due to higher nutrient concentrations in mWW, greater quantities of nutrients were taken up in mWW to produce the same percentage removal value as that in synWW. The potential for the UHCC0027 isolate to meet strict nutrient removal requirements in treating local municipal wastewater under 3% CO_2 was clearly demonstrated by the third day, where almost all NH_4^+-N and PO_4^{3-}-P had been consumed.

Under atmospheric CO_2, UHCC0027 demonstrated significantly greater capacity for nutrient removal in mWW than in synWW (between days 2 to 7 of the experiment, Fig. 3C and D). This greater capacity

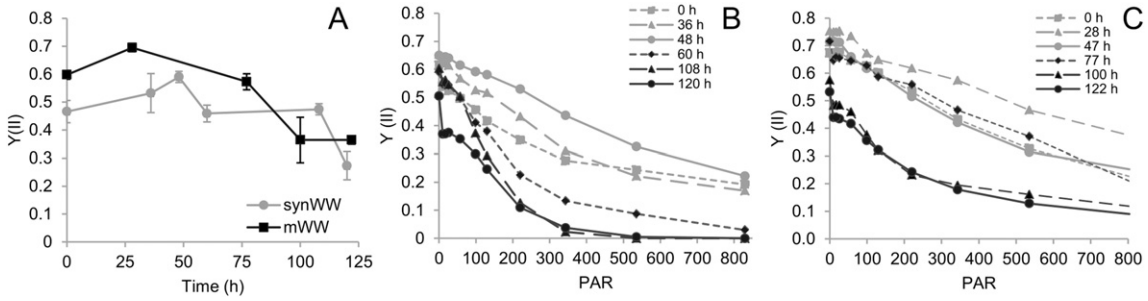

Fig. 4. Photosynthetic performance of UHCC0027. Effective PSII yield, Y (II), at different time points (h) of the 3% CO_2, high light, 16:8 light:dark experiment in synWW (grey circles, initial composition approximately 21 mg/L NH_4^+-N and 4 mg/L PO_4^{3-}P) and mWW (black squares, initial composition approximately 42 mg/L NH_4^+-N and 5 mg/L PO_4^{3-}P) (A) and light response curves of Y(II) over increasing intensities of photosynthetic active radiation (PAR) for synWW (B) and mWW (C) in the same experiment.

was likely a result of both the greater ammonium content and the additional organic carbon present in mWW, which had a dissolved COD of approximately 600 mg/L. Under atmospheric CO_2 conditions, the growth of UHCC0027 in mWW decreased after day 6. At day 8, there was a sudden appearance of Chl *a*, OD_{750} and nutrient removal in the blank (unseeded) sample. As Chl *a*, OD_{750}, and nutrient removal are all calculated relative to the blank, rapid declines in the performance of UHCC0027 in mWW are demonstrated at this time (Fig. 3). Microscopy observations performed throughout the experiment confirmed that at day 8, two morphologically distinct algae, indigenous to the mWW, were growing in the blank and, to a lesser extent, in the samples. Growth of the indigenous algae in the samples had increased relative to the blank by the final days (9 and 10) of the experiment, with resultant increases in all parameters except PO_4^{3-}-P removal. It is interesting to note that NH_4^+-N removal was only observed in the blank (unseeded) sample at day 7, and was thus also likely due to the indigenous mWW algae, rather than nitrifying bacteria that may have lacked the dissolved oxygen required to contribute.

3.3.5. Photosynthetic performance

The functional status of the photosynthetic apparatus in UHCC0027 cells grown in both synWW and mWW, under 3% CO_2 and the light:dark regime, was monitored using DUAL-PAM 100 to determine the effective quantum yield of Photosystem II, Y(II). In synWW, during the first 48 h, or 2 days, of the experiment, Y(II) increased from 0.47 (±0.04) to 0.59 (±0.00). At 60 h growth, Y(II) began a gradual decrease, finally reaching 0.27 (±0.05) at 120 h (5 days, Fig. 4A). Monitoring rapid light response curves of Photosystem II yield, Y(II), over gradually increasing light intensities (from 0 to 829 μmol photons m^{-2} s^{-1}) also demonstrated better photosynthetic performance and high light resistance in the cells grown in synWW for 36 and 48 h (1.5–2 days), compared to the control cells at 0 h (Fig. 4B). This correlates well with the intensive nitrogen and phosphorus uptake demonstrated by cells during the first 48 h (Fig. 3). Interestingly, after complete nutrient uptake, the cells were still growing exponentially, perhaps at the expense of internal nutrient reserves, to reach final OD_{750} values of 5–6. However, once cells were either lacking, or low on, nitrogen and phosphorus sources in the synWW medium (60–120 h), significant decreases in Y(II) values were observed. This was particularly clear under higher light intensities, implying light sensitivity and low photosynthetic capacity of the cells under low nutrient conditions (Fig. 4). On-line monitoring of Y(II) could therefore be useful as a rapid indicator, not only of the photosynthetic performance of the system, but also of the nutritional capacity of the growth medium.

UHCC0027 cells grown in mWW demonstrated higher Y(II) at 0 h (cells were pre-grown in higher nutrient media than those in synWW experiments) and a slower decrease in Y(II) under high light intensities than those grown in synWW (Fig. 4). These results and the general increase in high light tolerance observed for the cells grown in mWW (Fig. 4C) compared to synWW (Fig. 4B) may be due to greater intracellular reserves of nitrogen established during both the pre-growth period and the course of the experiment.

3.4. Screening for neutral lipid content

The lipid contents of BODIPY (505/515) stained cells were compared using high-throughput flow cytometry. Algal strains demonstrated greater median fluorescence intensity (MFI) from stained lipids than did cyanobacterial strains, with alga UHCC0027 demonstrating the greatest lipid based MFI of 1389 (Fig. 5). Neutral lipid content as determined by flow cytometry reflected total lipid contents determined gravimetrically. UHCC0027 and *Chlorella* produced 23.45 (±0.61) and 19.49 (±1.18) percentage lipids per dry weight, respectively. The higher lipid content determined for both green algae are not surprising, given the role that neutral storage lipids have been demonstrated to play in algal cell metabolism (see, for example, [7]). However, our study was performed after only 1 week of growth, where cells should be replete of nutrients (refer Fig. 1) and thus, the disparity between functional and storage lipids minimised. Given the nitrogen replete, low light, and atmospheric CO_2 conditions that cells were grown under, we expect the lipid content of these algae to be improved through the optimisation of growth conditions, whereby increases in lipid accumulation of up to 2–3 fold might be expected under conditions of nitrogen deprivation [41].

The successful staining of algal lipid droplets using BODIPY dye was confirmed using Confocal Laser Scanning Microscopy (CLSM). Cyanobacterial cells also demonstrated BODIPY fluorescence under CLSM, as has recently been demonstrated in the cyanobacterium *N. punctiforme* [13]. However, CLSM BODIPY fluorescence was observed to a lesser extent in cyanobacteria than in algae and required the evaluation of several fields of view for confirmation (Fig. 6). Flow cytometry provided more sensitive detection of BODIPY fluorescence, with the fluorescence signal of tens of thousands of cells collected in a matter of minutes. This sensitivity also resulted in the detection of non-specific signals, or noise. Data processing based on the methods of [22] separated cell-based flow cytometry signals from non-cellular fluorescent signals (due to dye precipitation) and from those resulting from debris. However, preliminary experiments indicated that processing was not successful in separating FITC channel fluorescence demonstrated by cyanobacterial strain 0TU37S4. Cells of this cyanobacterium grow covered with a thin mucilaginous layer when cultured in the laboratory for long periods of time [22]. This mucilaginous layer likely contributed to the fluorescent signal detected. Washing cells twice in 1 × PBS prior to staining and flow cytometry decreased the signal of 0TU37S4 to fall within the range of the other cyanobacteria. Thus, the difficulty in distinguishing 'real' lipid signal from that of mucilage or other extracellular substances should be considered when using BODIPY staining coupled with flow cytometry for the rapid identification

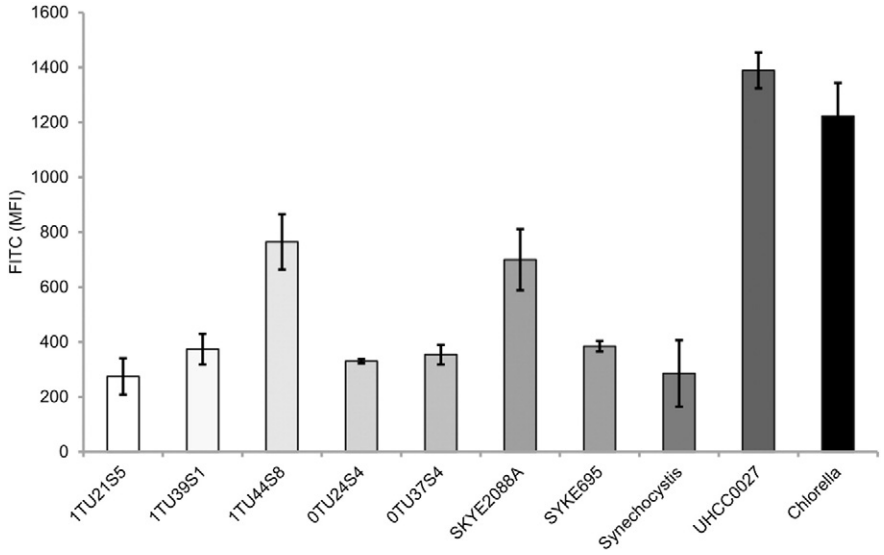

Fig. 5. Cellular neutral lipid contents of cyanobacteria and algae, as determined by BODIPY staining and flow cytometry detection (488 nm excitation laser and 530/30 nm band-pass filter). median fluorescence intensity (MFI) is plotted from 10,000 events (cells).

of high lipid producing strains. Despite this difficulty, the rapid screening process was useful in identifying the high lipid accumulation of *Chlorella* and UHCC0027 algae and for highlighting the carbon excretion potential of cyanobacterial strain 0TU37S4, which we will continue to study for its industrial potential.

3.5. Identification of strain UHCC0027

Strain UHCC0027 was the native isolate demonstrating the best potential for nutrient removal, biomass generation, CO_2 sequestration and lipid accumulation and thus is of great interest to our future studies. The identification of this isolate, from the coastal waters of Tvärminne (SW Finland), was initiated using standard 18S and ITS1 primers from literature. Of these, only the primer pair SSU1/SSU2 of [23] produced sequence data from the desired regions. Thus, primers were subsequently designed based on homologues of the initial PCR product, resultant sequences ensured good coverage (approximately 3.4 KB) of the 18S and ITS1 regions.

Sequences of 18S and ITS1 regions of UHCC0027 were used to collect the closest homologues from the NCBI GenBank database. Phylogenetic analysis placed UHCC0027 in a clade populated by the Scenedesmaceae family, with the closest genera being *Scenedesmus* and *Desmodesmus* (Fig. 7). The phylogenetic distance between UHCC0027 and the closest sequence is not negligible, demonstrating the novelty of the UHCC0027 strain, relative to algal sequences currently available in the GenBank database.

4. Conclusions

The value of screening native isolates was demonstrated by the identification of a native Scenedesmaceae isolate which outperformed cyanobacterial isolates and control strains in phosphate removal for

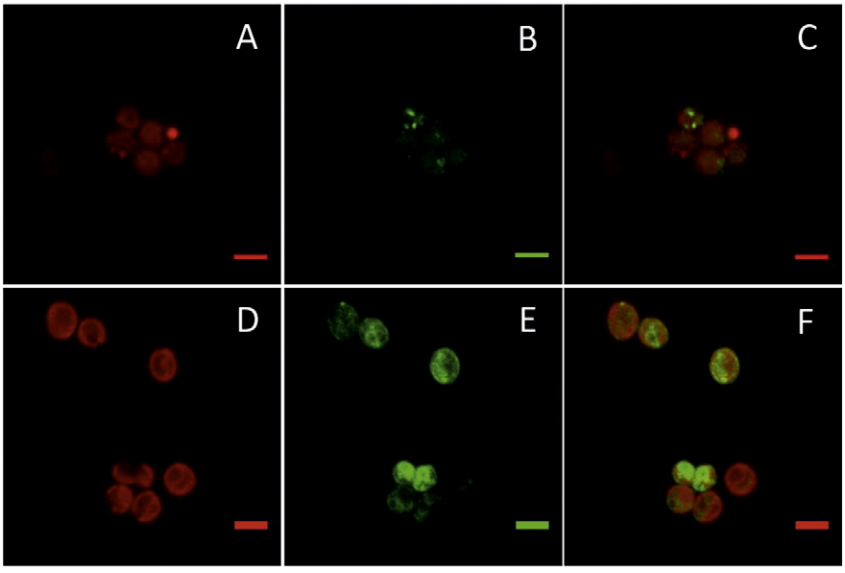

Fig. 6. Fluorescent photomicrographs of 1TU44S8 cyanobacterial cells (top, A–C) UHCC0027 algal cells (bottom, D–F). Chl autofluorescence is red (A & D) and lipids are stained green with BODIPY 505/515 (B & E). Image overlays are also presented (C & F). Scale bars indicate 5 μm.

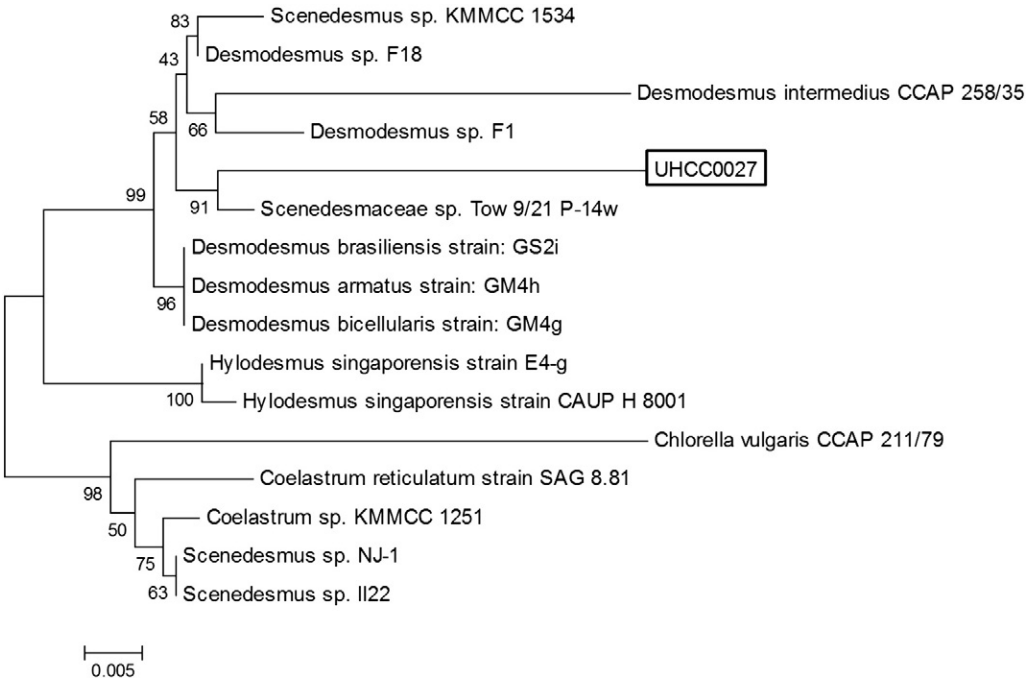

Fig. 7. Phylogenetic tree demonstrating the closest relatives of strain UHCC0027 based on BLAST searches of 18S rRNA and ITS1 genes (*Chlorella vulgaris* is included for reference). Numbers indicate branching confidence values, bar shows evolutionary distance in nucleotide substitutions per site. Scale bar represents 0.05% divergence.

wastewater treatment, biomass accumulation rates, and neutral lipid production. This native alga responded rapidly to process optimisation achieved by the addition of 3% CO_2, resulting in significantly faster biomass accumulation and the removal of 100% of ammonium and 90% of phosphate in only 2 days. The performance of the native Scenedesmaceae isolate was further tested in municipal wastewater, where it demonstrated more rapid growth than in synthetic media. Overall, this work presents the possibility, and foundation, for an integrated wastewater treatment and biofuel production platform featuring a native algal isolate.

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