Screening microalgae isolated from urban storm- and wastewater systems as feedstock for biofuel

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Exploiting microalgae as feedstock for biofuel production is a growing field of research and application, but there remain challenges related to industrial viability and economic sustainability. A solution to the water requirements of industrial-scale production is the use of wastewater as a growth medium. Considering the variable quality and contaminant loads of wastewater, algal feedstock would need to have broad tolerance and resilience to fluctuating wastewater conditions during growth. As a first step in targeting strains for growth in wastewater, our study isolated microalgae from wastewater habitats, including urban stormwater-ponds and a municipal wastewater-treatment system, to assess growth, fatty acids and metal tolerance under standardized conditions. Stormwater ponds in particular have widely fluctuating conditions and metal loads, so microalgae from this type of environment may have desirable traits for growth in wastewater. Forty-three algal strains were isolated in total, including several strains from natural habitats. All strains, with the exception of one cyanobacterial strain, are members of the Chlorophyta, including several taxa commonly targeted for biofuel production. Isolates were identified using taxonomic and 18S rRNA sequence methods, and the fastest growing strains with ideal fatty acid profiles for biodiesel production included *Scenedesmus* and *Desmodesmus* species (Growth Rate (d^{-1}) > 1). All isolates in a small, but diverse taxonomic group of test-strains were tolerant of copper at wastewater-relevant concentrations. Overall, more than half of the isolated strains, particularly those from stormwater ponds, show promise as candidates for biofuel feedstock.

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Abstract

Exploiting microalgae as feedstock for biofuel production is a growing field of research and application, but there remain challenges related to industrial viability and economic sustainability. A solution to the water requirements of industrial-scale production is the use of wastewater as a growth medium. Considering the variable quality and contaminant loads of wastewater, algal feedstock would need to have broad tolerance and resilience to fluctuating wastewater conditions during growth. As a first step in targeting strains for growth in wastewater, our study isolated microalgae from wastewater habitats, including urban stormwater-ponds and a municipal wastewater-treatment system, to assess growth, fatty acids and metal tolerance under standardized conditions. Stormwater ponds in particular have widely fluctuating conditions and metal loads, so microalgae from this type of environment may have desirable traits for growth in wastewater. Forty-three algal strains were isolated in total, including several strains from natural habitats. All strains, with the exception of one cyanobacterial strain, are members of the Chlorophyta, including several taxa commonly targeted for biofuel production. Isolates were identified using taxonomic and 18S rRNA sequence methods, and the fastest growing strains with ideal fatty acid profiles for biodiesel production included *Scenedesmus* and *Desmodesmus* species (growth rate (d^{-1}) > 1). All isolates in a small, but diverse taxonomic group of test-strains were tolerant of copper at wastewater-relevant concentrations. Overall, more than half of the isolated strains, particularly those from stormwater ponds, show promise as candidates for biofuel feedstock.

Introduction

As the earth's human population increases, world energy demands and reliance upon fossil fuels has continued to rise. Consequently, global carbon emissions, including green-house gases, have increased and contributed to global climate warming. The need to move to fossil-fuel alternatives is not only necessary for addressing anthropogenic climate change, but also depleting world energy-stores (Hook et al. 2013). Algal biofuels in particular have been identified as an exceptional source of carbon neutral, renewable energy (Sharif Hossain et al. 2008, Schenk et al. 2008, Clarens 2010). Their high photosynthetic efficiency, biomass production, and ability to accumulate relatively large amounts of triacylglycerides (TAG) for conversion to fatty acid methyl esters (FAME) have made them a desirable alternative to land-plant biofuel crops. Unlike first and many second-generation biofuel feedstocks, microalgae can be cultivated using saline, brackish or wastewater streams on non-arable land. Additionally, algal feedstock can be harvested batch-wise throughout the year from industrial-scale photobioreactors, reducing land, nutrient, and fresh water requirements (Guischina et al. 2006, Graef et al. 2009). The success and economic viability of an microalgae-based biofuel industry will depend on several factors, including the selection of robust strains that exhibit exceptional growth rates, suitable biofuel lipid profiles, resistance to disease and predation, and tolerance to a wide range of environmental parameters (Griffiths and Harrison 2009). Tolerance to environmental conditions is an important, but often over looked characteristic when bioprospecting for biofuels, and offers several benefits such as ease of cultivation and resiliency.

Griffiths and Harrison (2009) outlined a framework in the selection of appropriate algal strains. It identified the selection of fast-growing strains optimized for local climatic conditions as a fundamentally important quality. They also argued that lipid content as a key characteristic

46 may not be as critical as previously thought, because fast growth encourages high biomass
47 productivity, and high biomass density can increase yield-per-harvest volume. Research to
48 increase growth rates and lipid production has identified optimal temperatures, pH, photoperiods
49 and nutrients for several species (Lv et al. 2010, Sforza et al. 2012, Adams et al. 2013). These
50 parameters, however, are dissimilar among strains and require individual optimization.
51 Moving forward, it is recognized that bioprospecting for biofuel microalgae will require
52 assessments of individual and mixed-cultures of strains (Zhou et al. 2011). Additionally, ideal
53 candidate strains may need an inherent tolerance to sub-optimal growth conditions, since
54 wastewater from a variety of sources may be the most economically and environmentally
55 feasible growth medium. As a first step in assessing temperate-climate microalgae for their
56 potential as biofuel feedstock, we aimed to isolate and culture strains from several engineered
57 wastewater systems that included urban stormwater ponds and a municipal wastewater system.
58 These environments were targeted because they are known to support algal communities, but
59 also experience fluctuating conditions that include variation in contaminant loads and residence-
60 time.
61 For example, we know that stormwater ponds typically have elevated metal
62 concentrations (Campbell 1994; Marsalek and Marsalek 1997; Karlsson et al. 2010), including
63 the ponds in our study (Vincent and Kirkwood 2014). It also has been well documented that
64 municipal wastewaters have elevated levels of metals and other contaminants (Chambers et al.
65 1997; Gagnon and Saulnier 2003; Wang et al. 2005; Principi et al. 2006). Hence, these
66 engineered environments were considered promising source habitats for microalgae with
67 inherent tolerance to fluctuating conditions and contaminant loads, including metals. Overall, the
68 primary goal of this study was to elucidate if certain algal taxa from these wastewater source-

locations possessed growth, fatty acid, and metal-tolerance traits suitable for biofuel-feedstock production.

Materials and Methods

Sample Collection and Strain Isolation

Three 1-L water samples were collected from nine urban stormwater ponds and two natural reference ponds across Durham Region, Ontario, Canada between June and August, 2011. All of these ponds have been previously described using a similar numbering scheme in Vincent and Kirkwood (2014). In June, 2011, 3-L of municipal wastewater was collected from each of three locations along the secondary treatment system of the Corbett Creek activated sludge Water Pollution Control Plant in Whitby, Ontario, Canada. Additionally, several litres of water were collected from Lake Ontario at a single nearshore location in Whitby, Ontario to expand the isolations from natural, local habitats.

Sub-samples of all collected water were transferred to sterile 250-mL Erlenmyer flasks stoppered with cotton bunts on a light bank table (12:12 dark/light cycle) in triplicate. Upon confirmation of algal growth by visual inspection, 10-mL sample aliquots were transferred to 250-mL Erlenmyer flasks and enriched with 90-mL of two defined media recipes: BG11 (Rippka et al. 1979) modified by reducing $NaNO_3$ by one-tenth stock concentration; and CHU10 (Stein et al. 1973). Two media types were employed to broaden isolation success for a diversity of strains, and both had nitrogen and phosphorus concentrations on par with ranges typically found in the municipal-wastewater system of our study (unpublished data) and stormwater ponds (Vincent and Kirkwood 2014). F2 vitamin mixture (Guillard 1973) was added by syringe filter (Progene) to both media types after autoclaving. Isolation of unialgal strains was accomplished by methods

92 outlined by Andersen and Kawachi (2005). In brief, mixed-culture samples were serially diluted,
93 1 in 10 with sterile millipore water and spread plated on to 1.5% agar plates. Visible and distinct
94 colonies were pegged with sterile loop and re-cultured in BG11 or CHU 10 media and examined
95 microscopically to verify they were a single strain throughout the study period,with re-culturing
96 every three weeks. Isolated strains were observed using an EVOS xl core inverted microscope at
97 400X to ensure unialgal status, and subsequent sequencing of strains confirmed this. Tentative
98 taxonomic strain identification was assigned based upon morphological descriptions in Sheath
99 and Wehr (2003).
100
101 *Growth Assays*
102 Growth assays were conducted under standardized conditions to determine growth rate
103 and generation time for all isolates. A 10-mL inoculum of exponentially-growing stock-culture
104 was aseptically introduced to 90-mL of sterile isolation media and grown in cotton-stoppered
105 flasks on a cool-white fluorescent plate-glass light table (20-22 ˚C, 12:12 dark/light cycle) and
106 shaken by hand once a day. Algal cell concentration was determined by microscopic
107 enumeration using a Brightline haemocytometer on a VistaVision compound light microscope at
108 100X magnification. The optical density of 1-mL samples was measured at 550nm every 24-hrs
109 for a seven-day period using the Genesys 10S UV/VIS Spectrometer. These data were used to
110 construct growth curves for each algal strain. Specific growth rate (μ), and generation time (T_h)
111 were calculated as per Guillard (1973) over a period of exponential growth.
112
113 *Antibiotic Treatment of Algal Isolates*

Great care was taken to attain axenic cultures to remove the influence of bacteria on FAME profiles (Stemmler et al. 2016) using a modified antibiotic treatment method adapted from Jones et al. (1973). Isolated strains were first treated with a serial dilution of antibiotics (a combination of Penicillin G sodium salt and Streptomycin sulfate) (Bioshop Canada) for an exposure period of 24 hours. Algal colonies were selected from the lowest antibiotic concentration that had no bacterial growth. Aliquots of the exposed strains were transferred to fresh media, with no antibiotics, after 24 hours, followed by pour plate preparation, 1% peptone and 1.5% agar, to evaluate bacterial contamination in addition to microscopic observation. Strains deemed to be axenic were monitored by this method once a month to ensure axenic status was sustained. Strains that were found to be non-axenic were re-treated, but a few non-axenic strains were classified as such if bacterial contamination could not be removed. Only axenic strains were assessed for fatty acid analyses.

DNA Extraction, Amplification, and Sequencing

Genomic DNA of isolated strains was extracted using the commercial kit UltraClean Plant DNA Isolation kit (MoBIO, USA). Cell lysis was achieved by chemical, SDS and mechanical means using a Vortex Genie with horizontal microtube attachment (Scientific Industries, USA). Bead tubes provided by the extraction kit were subjected to three, 5-minute bead beating sessions at 3200 rpm. Samples rested in the tubes on ice for 5 minutes between bead beating sessions. Upon completion of cell disruption, 20 µL of RNase A (Geno Technology, USA) was added and vortexed gently for 10 minutes. Extracted DNA was quantified at 260/280nm by UV-Spectrophotometry (Gensys 10S UV/VIS) and frozen at -20°C or kept at 4°C until PCR amplification.

The 18S rRNA forward primer NS1 (White et al. 1990) and reverse primer ITS2 (White et al. 1990) were used to prime PCR at a concentration of 1.0 µmol. Reactions were carried out using Illustra Ready-To-Go PCR beads (GE Healthcare, USA) in a final volume of 25 µL. The PCR reaction was performed using the T100 Thermal cycler (BIORAD, Canada) with the following cycle parameters: initial denaturation 96°C for 3 min, annealing at 51°C /1 min, and extension at 72°C for 1.30 min. This was followed by 29 cycles of denaturation at 93°C /1.30 min, annealing at 51°C for 1.30 and extension at 72°C for 1.30 min, followed by a final extension step at 72°C for 5 min. Strains that would not amplify by this method were amplified using the forward NS1 primer and reverse 18L primer (Hamby and Zimmer 1991), along with a touchdown PCR cycle with annealing beginning at 61°C and reducing by 2°C every 2 cycles until 51°C was reached. Gel purification of PCR bands was carried out using the Wizard SV Gel and PCR Clean-Up System (Promega, USA). Modifications were made to the general protocol with the addition of an extra rinse of the column with membrane wash-solution as well as an additional 1-minute spin at 16000 x g with the lid off to aid in evaporation of wash solution. Extracted bands were sent to the Génome Québec Innovation Centre, McGill University, Canada for Sanger sequencing.

Sequencing results were locally aligned using the NCBI Nucleotide BLAST and closest matches assigned based on sequences from the NCBI database. BLAST matches that aligned closely, between 90-99% with culture strains, were selected and added to sequence comparisons in addition to more distantly related species within each family. Only NS1 region sequences greater than 400 base pairs (bp) were included in further phylogenetic analyses. These sequences were deposited into the NCBI database and accession numbers for each sequence were acquired.

159 Phylogenetic tree reconstruction was performed on the Phylogeny.fr platform (Dereeper
160 et al. 2008; Dereeper et al. 2010), which comprised a series of steps in the analysis. Sequences
161 were aligned and configured with the highest accuracy using MUSCLE (v 3.8.31) (Edgar 2004).
162 After alignment, ambiguous regions containing gaps or poorly aligned regions were removed
163 with Gblocks (v0.91b) using the following parameters: minimum length of a block after gap
164 cleaning=10; no gap positions were allowed; all segments with contiguous non-conserved
165 positions bigger than 8 were rejected; and minimum number of sequences for flank position was
166 85% (Castresana 2000). The phylogenetic tree was reconstructed using the neighbour-joining
167 method (Saitou and Nei 1987, Gascuel 1997) implemented in the BioNJ program with 1000
168 bootstrap replicates (Felsenstein 1985; Elias and Lagergren 2007). The analysis involved 45
169 nucleotide sequences with an average sequence length of 735 bp. Graphical representation and
170 editing of the phylogenetic tree was performed with TreeDyn (v198.3) (Chevenet et al. 2006).
171
172 *Fatty Acid Methyl Esters (FAME) Analysis*
173 To avoid possible confounding effects of bacteria, only axenic isolates (thirty-four in
174 total) were subjected to fatty acid analysis. Isolated strains were grown to stationary phase under
175 previously described growth conditions and 10-mL subsamples were transferred to 14-mL glass
176 tubes with lids and centrifuged at 16000 rpm (Sorval ST16, Thermo Scientific, USA) for five
177 minutes. Pelleted biomass was frozen at -20°C for 48 hours followed by 24-48 hrs of
178 lyopholization with a modulyoD freeze dryer (Thermo Scientific, USA). A direct method of
179 transesterfication of algal fatty acids was performed as per O'fallon et al. (2007). A 100 ppm
180 nonadecanoic acid methyl ester C19:0 (Sigma Aldrich, Canada) internal standard was added to
181 each sample followed by analysis by Gas Chromatography/Mass Spectrometry (GC/MS).

Methylated fatty acids in hexanes were analyzed on a Varian 450 gas chromatographer with HP-5ms Ultra Inert GC Column, 30 m x 0.25 mm x 0.25 µm, (Agilent J&W). A split ratio of 10:1 was used for 3.5 min with a flow rate of 1.0 mL/min, helium carrier gas. The oven temperature was held at 135 °C for 4 minutes and increased by 4 °C/min until 250 °C, where it was held for 10 minutes until run-end. A Varian 240 Ion Trap Mass Spectrometer detector was used and fame peaks were identified based upon spectral comparison and retention times to FAME standards: BAME and Supleco C8:0-C24:0 (Sigma Aldrich, Canada). A negative control was included in the FAME process as a quality measure and analyzed under identical sample conditions to ensure peaks obtained were attributable to microalgae samples.

To assess the biofuel potential of the lipid content measured for each isolate, several measures of biodiesel quality were determined using the methodologies and equations outlined in Ramos et al. (2009) and Nascimento et al. (2013). ASTM Standard D675 for biodiesel quality requires a minimum Cetane Number (CN) of 47 and a maximum Iodine Value (IV) of 120.

Copper Toxicity Bioassays

Five isolates representing diverse taxonomic genera were further characterized to assess their inherent tolerance to copper, a particularly toxic metal to microalgae and a metal that is commonly found in industrial and municipal wastewaters. Genera selected include: *Scenedesmus, Monoraphidium, Chlorella, Selenastrum* and *Microcystis*. The Canadian Phycological Culture Collection strain *Chlorella kesslerii* CPCC266 was also tested as it is a commonly used bioassay reference-strain. A standard 72-hour static growth response bioassay was used to assess inherent tolerance to copper. The assay methodology was based on the protocol established by Environment Canada (2007). Prior to commencing the bioassays, all

glassware, tips, and applicable materials were acid-washed in a 10% HCL solution for a period of 24 hours followed by triplicate rinse with Milli-Q™ filtered water. A stock Copper (II) Sulfate Pentahydrate (32 mg L^{-1} Cu) solution was prepared with a solution of CHU 10 media and sterilized by autoclave. The stock solution was used to aseptically prepare copper concentrations of 0.01, 0.1, 0.32, 1.0 and 3.2 parts per million (ppm) Cu, diluted with sterile CHU 10 media. All concentrations and control cultures were prepared in triplicate to a final volume of 25-mL in 50-mL Erlenmyer flasks. Innocula (1-mL) from exponential phase cultures were added to three replicate flasks with each test concentration and maintained under the same growth conditions in a controlled environment incubater with shaker table (Algaetron AG130-ECO) at 22°C $^{+/- 2°C}$ under 12h light/dark cycles at 150 mmol photons m^{-2} s^{-1} and 200 rpm. The pH of each replicate was monitored daily using an Oakton pH/Ion 510 bench meter. Fluorescence measurements of chlorophyll *a* were taken at 24-hr intervals using an Aquafluor Hand Held fluorometer, whereby the chlorophyll *a* solid secondary standard (Turner Designs, USA) was used as a qualitative calibration tool for chlorophyll *a* measurement. The growth rate was calculated as previously described and data were normalized to percent-control. Growth-rate data were also used to calculate percent inhibition:

$$\% I = \frac{C - X}{C} \times 100$$

Where C = average control growth-rate, X = average test-concentration growth-rate.

Results

In total, forty-three algal strains were isolated from all habitat types. Tentative taxonomic assignments were given to all isolates and, with the exception of one cyanobacterial strain (*Microcystis* sp. Sp21.01), NCBI BLAST searches of partial 18S rRNA sequences were

performed (Table 1). All eukaryotic isolates are members of the algal division Chlorophyta, which is a diverse group of green microalgae. Taxonomic assignment and BLAST matches were fairly congruent, but there were several exceptions. All isolates were initially characterized for growth-rate under standardized conditions (Table 1), and for all strains, exponential growth was most commonly observed to last up to three days. Fastest growing strains came from stormwater ponds and included *Scenedesmus sp* Sp19.011 and *Desmodesmus* sp. Sp19.15, which doubled their biomass in just over 13 hours. In contrast, the slowest growing strains *Scenedesmus* sp. Sp21.12 and *Chlorella* sp. Sp21.20 had generation times between 40-42 hours. Over 60 % of the strains were found to have a generation time less than 24 hours (Table 1).

A subset of twenty-seven isolates with NS1sequences greater than 400 bp were used with eighteen NCBI database-taxa to reconstruct an unrooted neighbour-joining phylogenetic tree (Fig. 1). All strains included in the tree belong to the Chlorophyceae and Trebouxiophyceae classes. Tree topology distinctly groups isolates into families and genera. In total, the tree demarcates study-isolates into seventeen unique phylotypes based on branch lengths ≤ 0.001 base-pair substitutions per site. Most isolates had sequence matches with taxa from the BLAST database, with the exception of only a few including: *Monoraphidium sp*. Sp19.015, *Scenedesmus sp*. Sp19.011, *Desmodesmus sp*. Sp19.15 and *Chlamydomonas sp*. SpU9.

FAME results for thirty-four axenic isolates identified seven different fatty acids, including saturated (lauric acid (C12:0) and palmitic acid (C16:0)), monounsaturated (oleic acid (C18:1)) and polyunsaturated (linoleic acid (C18:2), hexadecatetraenoate (C16:4(n-3) and eicsoatetraenoic acid (C20:4)) forms. Palmitic and oleic fatty acids were the predominant lipid components found in all strains. Several strains including *Chlamydomonas* sp. SpU9, *Chlamydomonas* sp. Sp19.40, *Ankistrodesmus* sp. LO47 and *Chlorella* sp. LO51 had a large

251 percentage (70-90%) of their total FAME lipid as palmitic acid. *Desmodesmus* sp. WW39 and
252 *Scenedesmus* sp. Sp16.34 had oleic acid greater than 80% of their total FAME lipid profile.
253 *Chlorococcum* sp. Sp17.022 was the only strain observed to have oleic acid C18:1 present in
254 both cis- and trans- forms. One of the most unique profiles belongs to *Chlorella sp*. Sp21.02,
255 which was found to have 53% of its lipids in polyunsaturated forms (Fig. 2). There was no
256 apparent distinction of FAME profiles among strains isolated from different source-locations
257 (see supplemental information).

258 To determine if there was any congruence among strains with respect to lipid
259 composition and phylogenetic relatedness, the FAME profiles of strains sharing a phylotype with
260 at least one other strain were compared using hierarchical cluster analysis (Fig. 3). The Bray-
261 Curtis similarity dendrogram grouped strains of the same phylotype as being most similar based
262 on their FAME profile. The cophenetic correlation coefficient of 0.87 indicates that the
263 dendrogram adequately preserved the pairwise distances among strains. Bootstrap scores were
264 mixed, but were moderate to strong for most nodes in the dendrogram. Strains *Scenedesmus sp*.
265 Sp12.36 and *Scenedesmus sp*. LO48 are the same phylotype and had the most similar FAME
266 profiles (>95% similarity). In contrast, *Ankistrodesmus sp*. LO47 and *Ankistrodesmus* sp. Sp1.50,
267 which are the same phylotype, were only about 55% similar based on their FAME profiles. Even
268 so, they were grouped as most similar among isolates in the cluster dendrogram (Fig. 3).

269 With respect to biodiesel quality, twenty-eight isolates had a CN greater than 47, and
270 thirty isolates did not exceed an IV of 120 (Table 2). In combination, twenty-eight isolates had
271 acceptable CN and IV values according to the ASTM standard. The degree of unsaturation
272 among isolates was quite varied, ranging from very low (10.6) for *Chlorella sp*. Sp23.13 to very
273 high (120) for *Ankistrodesmus sp*. Sp1.50. Long-chain saturation factor also varied among

274 isolates, where isolate *Scenedesmus* sp. Sp16.34 had the lowest value (0.55) by an order of
275 magnitude, and *Chlorella* sp. Sp23.13 had the highest value (8.94). The Saponification Value
276 was less varied among strains, where most had values varying from 200-210. Strain
277 *Chlamydomonas sp*. SpU9 had a notably high SV of 241. The majority of isolates had negative-
278 integer values for Cold Filter Plugging Point. *Scenedesmus sp*. Sp16.34 in particular, had a CFPP
279 as low as -15 °C. In contrast, Sp23.13 had a relatively high CFPP at 12 °C.
280 To assess the inherent tolerance of algal isolates to the common wastewater contaminant
281 copper, five representative-strains from a variety of taxonomic groups were subjected to static
282 growth-response bioassays. The cyanobacterial strain *Microcystis* sp. Sp21.01 was the least
283 tolerant to copper, having complete growth inhibition at 0.32 $mg \cdot L^{-1}$ or higher. The two
284 stormwater pond isolates *Monoraphidium* sp. Sp17.38 and *Selenastrum* sp. Sp14.35 had
285 significantly higher (Student's t-test $p<0.05$) tolerance to 0.32 $mg \cdot L^{-1}$ copper than the reference
286 strain *Chlorella kesslerii* CPCC26. The most tolerant strain was *Selenastrum sp*. Sp14.35, which
287 had the highest growth rate at 1 $mg \cdot L^{-1}$ copper. No strains could grow at the highest test
288 concentration 3.2 $mg \cdot L^{-1}$. At municipal-wastewater relevant concentrations of copper, all test
289 strains, with the exception of *Microcystis* sp. Sp21.01, had minimal to no growth inhibition at the
290 highest copper concentration 0.1 $mg \cdot L^{-1}$ (Table 3).
291
292 **Discussion**
293 *Diversity of algal isolates*
294 All isolated algal strains (with the exception of one cyanobacterial isolate) belong to the
295 Chlorophyta, a class of algae that includes taxa commonly used in algal biofuel research and
296 application (Mata et al. 2010). In particular, *Chlorella sp*. and *Scenedesmus sp*. are the most

297 commonly used for biofuel feedstock, and our study isolated several strains from each of these
298 genera. Chlorophyte taxa such as the strains from our study are easy to culture, which in part, is
299 likely why they are common in biofuel studies. Yet, these taxa have also been shown to be
300 relatively good lipid producers (Mata et al. 2010), which increases their utility as feedstock in
301 biodiesel production. As such, all source locations in this study, and in particular stormwater
302 ponds and the municipal wastewater treatment plant, were fruitful habitats for isolation of lipid-
303 rich strains suitable for biodiesel production.

304 Several discrepancies between taxonomic assignment and BLAST match (Table 1) was
305 not entirely unexpected as this can be a common scenario. Though many chlorophyte species
306 exhibit type-features, such as spine and coenobium formation, culture condition will often affect
307 these characteristics used in identification. Morphological heterogeneity also makes algal
308 identification challenging, so it is possible that some strains were misidentified. In contrast, 18S
309 rRNA sequencing can be useful in distinguishing phylotypes of morphologically similar strains
310 (Friedl, 1995, Lewis and McCourt, 2004). This was evident in the phylogenetic tree
311 reconstruction, which provided a clear resolution to generic assignment for isolates in the tree. In
312 most instances, morphological similarities of clade members were supported by genetic
313 homology presented in the tree organization. BLAST sequence searches for some of the more
314 closely related *Scenedesmus, Desmodesmus* and *Coleastrum* taxa often returned similar local
315 alignment matches for query cover and percent identity, making them difficult to confirm solely
316 based on closest-match results. Again, the phylogenetic tree topology provided useful
317 clarification of misidentifications found in the BLAST database. Sequence databases are known
318 to have a small percentage (~20%) of their sequence collection to be mislabelled or misidentified
319 (Bridge et al, 2003, Vilgalys, 2003), and algal systematics is currently in flux. As such, definitive

taxonomic and phylogenetic assignment for strains in this study will likely require further classification in the future.

Algal growth and fatty acid production

Rapid growth rates are an important characteristic of biofuel feedstock, since fast-growing strains would have increased yields in a shorter time-frame. According to Griffiths and Harrison (2009), the average doubling time for green microalgae is 24 h, which corresponds to a growth-rate (μ) = 0.69 d^{-1}. Of the forty-three isolates evaluated, the majority of strains were able to double their biomass in 24-hours or less (Table 1). Twelve isolates from our study could grow ≥ 1.0 d^{-1} (Table 1), which is considered to be a relatively fast growth-rate for microalgae in general, and thus a desirable trait for biofuel-feedstock. Many of the fastest growing taxa were from stormwater ponds and included closely related genera such as *Scenedesmus*, *Desmodesmus*, and *Ankistrodesmus* (Fig. 1). If these phylogenetically related taxa were to be targeted for isolation from other systems, our results from a subset of strains would suggest that they may have similar fatty acid profiles (Fig. 2), even for those of the same phylotype (Fig. 3). However, calculations presented in Table 2 show phylogenetically similar strains (e.g., Sp12.07 and Sp12.21) have differing biodiesel qualities. This finding concurs with the Culture Collection of Algae at Goettingen University (SAG) study that found similarities among fatty acid distribution patterns were only found at phyla and class levels (Lang et al. 2011). When accounting for all strains that were assessed for their fatty acid profiles (Fig. 2, Table 2), it is apparent that the diverse array of taxa, even from the same class, exhibited a broad range of variation in fatty acid composition. The observed variation in growth rate and fatty acid profile among strains in our collection supports the continued need to characterize microalgae at the strain-level, rather than targeting a certain family or genera, when bioprospecting for biofuel candidates.

Common fatty-acid esters found in manufactured biodiesel include: palmitic C16:0 (hexadecanoic) acid, stearic C18:0 (octadecanoic) acid, oleic C18:1 (9(Z)-octadecenoic) acid, linoleic C18:2 (9(Z),12(Z)-octadecadienoic) acid, and linolenic C18:3 (9(Z),12(Z),15(Z)-octadecatrienoic) acid (Knothe 2008). The dominant saturated fatty-acid in most strains from this study was C16:0 palmitic acid, and in many cases, represented approximately 50% of the total fatty-acid profile. The dominant unsaturated fatty acid in most strains was C18:1 oleic acid, and it too represented about 50% of the total fatty acid profile among several strains. Biofuels with high oleic acid content have been reported to have reasonable ignition quality, combustion heat, cold filter plugging point (CFPP), oxidative stability, viscosity, and lubricity (Chen 2012). Hu et al. (2008) confirm that fatty acids with carbon chain lengths from 16 to 18 units are ideal precursors for biodiesel production. Not only does carbon chain length provide a basis for deriving biodiesel, but specifically, the saturation versus unsaturation garners characteristics that make the fuel more versatile.

Potential for biodiesel production

Biodiesel derived from microalgae with more saturation provide a higher cetane number, would have lower NOx emissions, and have shorter ignition delay time (Cherisilp and Torpee 2012). Nevertheless, this can come at a cost since lower temperatures would cause saturated fatty- acids to solidify due to their high melting point (Doğan and Temur 2013). Unsaturated fatty-acids require less heating and are often liquids at room temperature, however, the higher the number of double-bonds, the more prone the fuel is to producing NOx emissions (Gopinath et al. 2010). Gopinath et al. (2010) claim that having a 50/50 blend of saturated and unsaturated fatty acids produces better thermal efficiency and reduces NOx emissions. Based on this biodiesel characteristic, many of the strains isolated in this study have desirable fatty-acid profiles

366 reflecting a ~50/50 saturated/unsaturated blend.

367 TAG commonly accumulate in many algal species as a storage product. As culture
368 resources are depleted and algal growth slows, so does the production of new membrane
369 compounds such as phosphoglycerides, glycosylglycerides and sterols. Under these conditions,
370 fatty-acid production is diverted to TAG synthesis (Guschina and Harwood 2006). This
371 metabolic shift results in a trade-off impacting biomass production. Griffiths and Harrison (2009)
372 reported that cultivation conditions focusing on biomass productivity instead of lipid production
373 per cell may ultimately be more beneficial and more efficient in increasing total lipid
374 productivity. Several fast growing isolates from this study (including *Scenedesmus* sp. Sp1.41,
375 *Scenedesmus* sp. Sp19.011, and *Desmodesmus sp*. Sp19.15) have ideal biodiesel properties based
376 on their Cetane Number, Iodine Values and Degree of Unsaturation (Table 2). Hence, these
377 strains may represent the best candidates for optimal lipid yields based on growth-rate alone.

378 *Algal copper tolerance*

379 Although a select, but diverse group, of isolates were tested for copper tolerance, all
380 chlorophyte strains exhibited high tolerance at wastewater-relevant concentrations (Table 3).
381 Thus it is plausible that many chlorophyte isolates in our collection would likely have some
382 inherent tolerance to copper if one extrapolates from the copper bioassay results. Others have
383 also recognized the higher tolerance thresholds of chlorophyte microalgae to wastewater
384 conditions, focusing primarily on *Chlorella* and *Scenedesmus* strains (Lau et al. 1995; Bhatnagar
385 et al. 2010; Ruiz-Marin et al. 2010). Interestingly, the only non-chlorophyte strain in our study
386 (*Microcystis*) was uniquely less tolerant to copper than the chlorophyte strains, even though it
387 was also isolated from a stormwater pond. It is also interesting that two of the stormwater pond
388 isolates *Monoraphidium* sp. Sp17.38 and *Selenastrum* sp. Sp14.35 were notably tolerant to

389 copper at concentrations orders of magnitude higher than their source stormwater ponds (Table
390 3). Therefore, these strains in particular may be ideal candidates for industrial-wastewater
391 feedstock since they are relatively fast growers (Table 1), have ideal fatty-acid profiles (Fig. 2
392 and Table 2), and have inherent tolerance to the algicidal-metal copper (Table 3).

393 *Conclusions*

394 Bioprospecting of algal strains for use in biofuel production is a relatively new trend.
395 Though our study is the first to target biofuel bioprospecting efforts in stormwater ponds and a
396 municipal wastewater system, the dominant taxa isolated, including *Chlorella, Scenedesmus, and*
397 *Desmodesmus,* are cosmopolitan in freshwater environments. Strains identified as the species
398 *Scenedesmus obliquuss* and *Chlorella leuteoiviridis* in blast matches were prevalent in both
399 stormwater ponds and the wastewater treatment facility, and they typically out-competed other
400 species during cultivation. Zhou et al. (2011) found similar results in isolations of microalgae
401 from wastewater sites in the United States, isolating several species of *Chlorella*. Past efforts to
402 isolate microalgae from various habitats including wastewater systems was mainly for
403 wastewater remediation. *Chlorella* and *Scenedesmus* are common taxa isolated for this purpose,
404 especially for nutrient removal (Tam and Wong 1989; Tam and Wong; 1990, Gonzalez et al.
405 1997; Woertz et al. 2009; Chinnasamy et al. 2010). Nutrient removal by microalgae cultivated in
406 wastewater would provide a value-added benefit during biofuel-feedstock production.

407 While the results presented here are only a first step in assessing algal strains from
408 wastewater habitats as biofuel-feedstock candidates, the ultimate goal is to establish and select
409 strains most tolerant to municipal and/or industrial wastewater conditions. The use of wastewater
410 to support algal biomass production is not a new approach, but it is for biofuel feedstock
411 production. Several studies have all concluded that there is great potential for algal biofuel

412 production using wastewater (Pittman et al. 2011; Bhatnagar et al. 2011; Christenson and Sims
413 2011). Yang et al. (2011) looked at the total water and nutrient requirements of microalgae-based
414 biofuels and found that biofuels sourced from wastewater decreased total water requirements by
415 90% in comparison to freshwater sourcing without recycling. What has not been investigated
416 explicitly is the viability of using wastewater on a continuous basis in a pond or photobioreactor
417 system growing single or mixed strains of microalgae. Broad variations can occur in wastewater
418 quality, in addition to fluctuations in toxic-metal constituents (Cole et al. 1989; Ahuwalia et al.
419 2007). In the case of municipal wastewater, the nutrient and contaminant content can vary daily
420 depending on the source-population. As such, future studies of biofuel microalgae, including
421 promising isolates from this study, would need to be assessed for their tolerance and resilience to
422 fluctuating wastewater conditions while maintaining an acceptable growth rate and lipid yield.

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Table 1(on next page)

Summary list of isolated strains.

Summary list of isolated strains and their associated isolation medium, source location, tentative taxonomic assignment and 18S rRNA sequence similarity with BLAST taxa. Growth rate and generation time under standardized growth conditions over 7-days are also included. With the exception of one cyanobacterial strain (*Microcystis* sp.) which has been listed because of its inclusion in the bioassay results, all eukaryotic strains were subjected to 18S rRNA sequencing using NS1 and ITS2/18L primers. NS = Strain not successfully sequenced, WWTP = Wastewater Treatment Plant, SWP = Stormwater Pond, RP = Reference Pond, Lake ON = Lake Ontario. See Vincent and Kirkwood (2014) for more information on stormwater and reference pond characteristics.

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Strain ID	Isolating Medium	Source Location	Taxonomic Assignment	Closest BLAST Match	Similarity (%)	Sequence Size (bp)	Growth Rate (d^{-1})	Generation Time (hrs)
Sp1.41	CHU10	SWP 1	*Scenedesmus* sp.	*Desmodesmus armatus KF673362*	99	1160	1.14	14.6
Sp1.43	CHU10	SWP 1	*Desmodesmus* sp.	*Desmodesmus intermedius* FR865701	98	1253	0.74	21.1
Sp1.44	CHU10	SWP 1	*Chlorella* sp.	*Chlorella* sp. KP262476	99	1580	0.75	22.2
Sp1.46	CHU10	SWP 1	*Dictyosophaerium* sp.	*Dictyosphaerium ehrenbergianum* GQ487213	99	474	0.69	24.0
Sp1.50	CHU10	SWP 1	*Ankistrodesmus* sp.	*Ankistrodesmus gracilis. AB917098*	98	1618	1.15	14.5
Sp1.52	CHU10	SWP 1	*Chlorella* sp.	*Mychonastes rotundus* GQ477053	83	343	0.97	17.2
Sp11.30	CHU10	SWP 11	*Scenedesmus* sp.	*Scenedesmus obliquus* FN298925	99	911	0.51	32.6
Sp12.07	BG11	SWP 12	*Chlorella* sp.	*Coelastrum astroideum* GQ375093	99	1518	0.55	30.2
Sp12.21	CHU10	SWP 12	*Chlorella* sp.	*Coelastrum astroideum* GQ375093	100	850	1.04	16.1
Sp12.36	CHU10	SWP 12	*Scenedesmus* sp.	*Acutodesmus obliquus AB917101*	99	1614	0.90	18.6
Sp13.17	CHU10	SWP 13	*Chlorella* sp.	*Mychonastes huancayensi* GQ477050	100	1445	0.99	17.7
Sp14.35	CHU10	SWP 14	*Selenastrum* sp.	NS	--	--	0.96	17.41
Sp16.26	CHU10	SWP 16	*Scenedesumus* sp.	*Scenedesmus* sp. FN298925	99	1431	0.90	18.6
Sp16.34	CHU10	SWP 16	*Scenedesmus* sp.	*Scenedesmus obliquus* KJ676128	99	714	1.10	15.1
SpU.9*	CHU10	SWP 16	*Chlamydomonas* sp.	*Chlamydomonas inflexa* FR865584	97	1143	0.59	28.1
Sp17.013	BG11	SWP 17	*Monoraphidium* sp.	*Monoraphidium convolutum* HM483515	99	1309	0.92	18.1
Sp17.022	BG11	SWP 17	*Chlorococcum* sp.	*Chlorococcum ellipsoideum* U70586	95	423	0.65	25.6

Sp17.25	CHU10	SWP 17	*Chlorella* sp.	*Chlorella luteoviridis* FR865678	99	1482	0.98	17.0
Sp17.38	CHU10	SWP 17	*Monoraphidium* sp.	*Monoraphiridium* sp. JN187941	98	873	1.12	14.9
Sp19.010	BG11	SWP 19	*Desmodesmus* sp.	*Desmodesmus communis* KF864475	99	1178	0.64	26.0
Sp19.011	BG11	SWP 19	*Scenedesmus* sp.	*Scenedesmus acuminatus AB037088*	99	1073	1.24	13.5
Sp19.015	CHU10	SWP 19	*Monoraphidium sp*	*Monoraphiridium contortum* AY846382	97	588	0.72	23.1
Sp19.15	CHU10	SWP 19	*Desmodesmus* sp.	*Desmodesmus pannonicus* FR865712	98	821	1.22	13.7
Sp19.40	CHU10	SWP 19	*Chlamydomonas* sp.	*Chlamydomonas debaryana* JN903975	99	651	1.10	15.1
Sp21.01	BG11	SWP 21	*Microcystis* sp.	--	--	--	0.69	24.0
Sp21.02	BG11	SWP 21	*Chlorella* sp.	*Chlorella luteoviridis* FR865678	99	718	0.80	20.7
Sp21.12	CHU10	SWP 21	*Scenedesmus* sp.	*Scenedesmus* sp. FR865732	99	1483	0.40	41.8
Sp21.14	CHU10	SWP 21	*Chlorella* sp.	*Chlorella luteoviridis* FR865678	99	449	0.75	22.1
Sp21.20	CHU10	SWP 21	*Chlorella* sp.	*Chlorella luteoviridis* FR865678	100	1336	0.42	39.8
Sp21.37	CHU10	SWP 21	*Ankistrodesmus* sp.	*Monoraphidium contortum* KM067465	94	626	1.10	15.1
Sp21.23	CHU10	SWP 21	*Desmodesmus* sp.	*Desmodesmus intermedius* FR865701	99	1245	1.07	15.6
Sp23.13	CHU10	RP 23	*Chlorella* sp.	*Micractinium inermum* KF597304	100	1005	0.55	30.3
Sp24.1	CHU10	RP 24	*Scenedesmus*sp.	*Scenedesmus* sp. FN298925	100	257	0.55	30.3
Sp24.05	BG11	RP 24	*Desmodesmus* sp.	*Desmodesmus pannonicus* FR865712	99	1158	0.50	33.2
LO47	CHU10	Lake ON	*Ankistrodesmus* sp.	*Monoraphidium griffthii.* AY846383	100	1454	0.74	22.5
LO48	CHU10	Lake ON	*Scenedesmus* sp.	*Scenedesmus obliquus* FR865738	100	1119	1.00	16.7
LO49	CHU10	Lake ON	*Chlorella* sp.	*Micractinium* sp. *AB9187105*	100	350	1.08	22.2
LO51	CHU10	Lake ON	*Chlorella* sp.	*Chlorella luteoviridis. FR865678*	100	585	0.58	29.1
WW3	CHU10	WWTP	*Chlorella* sp.	*Coelastrum microporum* JQ315528	98	497	0.63	25.3
WW5	CHU10	WWTP	*Scenedesmus* sp.	*Acutodesmus obliquus* KF144164	99	597	0.58	28.7
WW8	CHU10	WWTP	*Chlorella* sp.	*Chlorella luteoviridis.* FR865678	99	761	0.80	20.7
WW27	CHU 10	WWTP	*Chlorella* sp.	*Chlorella luteoviridis* FR865678	99	1304	0.77	21.7
WW39	CHU10	WWTP	*Desmodesmus* sp.	*Desmodesmus intermedius* KF673371	99	1572	0.66	25.4

*The source location for strain SpU.9 is either SWP 16 or SWP 17.

Table 2(on next page)

Biodiesel properties of algal isolates.

Biodiesel properties of algal isolates based on their fatty acid profiles. The ASTM Biodiesel Standard D675 requirement for Cetane Number is a minimum value = 47 and Iodine Value maximum = 120. Degree of Unsaturation is a weighted sum of the masses of monounsaturated and polyunsaturated fatty acids, and Long-Chain Saturation Factor is a weighted sum of long-chain fatty acids (C16, C18, C20, C22 and C24). Saponification value is equivalent to milligrams of potassium hydroxide required to saponify 1 g of oil.

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Strain ID	Cetane Number	Iodine Value	Degree of Unsaturation (wt %)	Long-Chain Saturation Factor (wt %)	Saponification Value (mg)	Cold Filter Plugging Point (°C)
Sp1.41	59.4	51.9	57.7	4.44	207	-3
Sp1.43	61.0	45.0	50	5.12	209	0
Sp1.44	53.7	74.3	66.9	4.9	207	-1
Sp1.46	58.0	56.3	62.5	4.01	210	-4
Sp1.50	18.9	207	120	2.37	215	-9
Sp1.52	55.4	67.0	60.4	4.51	209	-2
Sp12.07	44.6	108	76.7	4.11	211	-4
Sp12.21	57.4	59.7	56.6	4.1	207	-4
Sp12.36	55.3	68.7	68.6	3.45	206	-6
Sp14.35	60.3	48.3	53.7	4.69	208	-2
Sp16.26	59.1	53.7	59.8	4.02	206	-4
Sp16.34	45.6	110	104	0.55	200	-15
SpU9	65.6	13.3	37.3	8.26	241	9
Sp17.013	36.3	139	85.7	4.16	214	-3
Sp17.022	66.0	22.7	46.1	6.44	214	4
Sp17.25	61.3	42.9	47.6	6.02	210	2
Sp19.010	55.0	68.0	60.5	4.4	210	-3
Sp19.011	54.7	68.0	57.7	4.59	212	-2
Sp19.15	56.4	63.2	59.8	4.43	208	-3
Sp19.40	68.8	10.7	11.9	8.81	216	11

Sp21.02	25.3	186	118	3.51	206	-5
Sp21.12	58.1	56.6	56.6	4.71	208	-2
Sp21.14	55.0	68.5	62	5.15	208	0
Sp21.20	53.9	71.9	60	5.76	210	2
Sp21.37	60.7	46.5	51.7	4.83	208	-1
Sp23.13	69.1	9.60	10.6	8.94	216	12
Sp24.05	47.7	94.8	69	4.18	213	-3
LO47	56.1	61.1	42	7.24	215	6
LO48	53.9	73.9	71.3	3.44	206	-6
LO49	52.6	78.6	66.2	5.15	207	0
LO51	67.8	14.5	48.8	6.88	217	5
WW3	53.9	73.5	73.1	2.67	207	-8
WW39	49.5	93.0	90.6	1.45	203	-12
WW5	31.0	162	35.2	2.01	210	-10

Table 3(on next page)

Comparison of algal tolerance to copper.

Comparison of algal tolerance to the range of copper concentrations typically found in municipal-wastewater systems (Environment Canada 2001). Copper concentrations previously measured for source stormwater-ponds are also included (Vincent and Kirkwood 2014). Average percent-inhibition is reported for each test concentration: N/A = information not available.

Table 3. Comparison of algal tolerance to the range of copper concentrations typically found in municipal-wastewater systems (Environment Canada 2001). Copper concentrations previously measured for source stormwater-ponds are also included (Vincent and Kirkwood 2014). Average percent-inhibition is reported for each test concentration: N/A = information not available.

		Copper (% Inhibition)	
	Source Location		
Strain ID	**Copper (mg · L^{-1})**	**0.01 mg · L^{-1}**	**0.1 mg · L^{-1}**
Chlorella kesslerii CPCC266	N/A	0	0
Chlorella sp. Sp21.20	0.0021	5.25	5.84
Scenedesmus sp. Sp11.30	0.0035	0	0
Selenatrum sp. Sp14.35	0.0061	0	1.04
Monoraphidium sp. Sp17.38	0.0040	0	0.22
Microcystis sp. Sp21.01	0.0021	0.86	53.4

Figure 1(on next page)

Unrooted Neighbor-joining tree for 18S rRNA NS1-region sequences

Unrooted Neighbor-joining tree for 18S rRNA NS1-region sequences greater than 400 bp for algal isolates and related taxa from the NCBI database. Bootstrap scores are based on 1000 replicates. NCBI database accession-codes have been included with strain names.

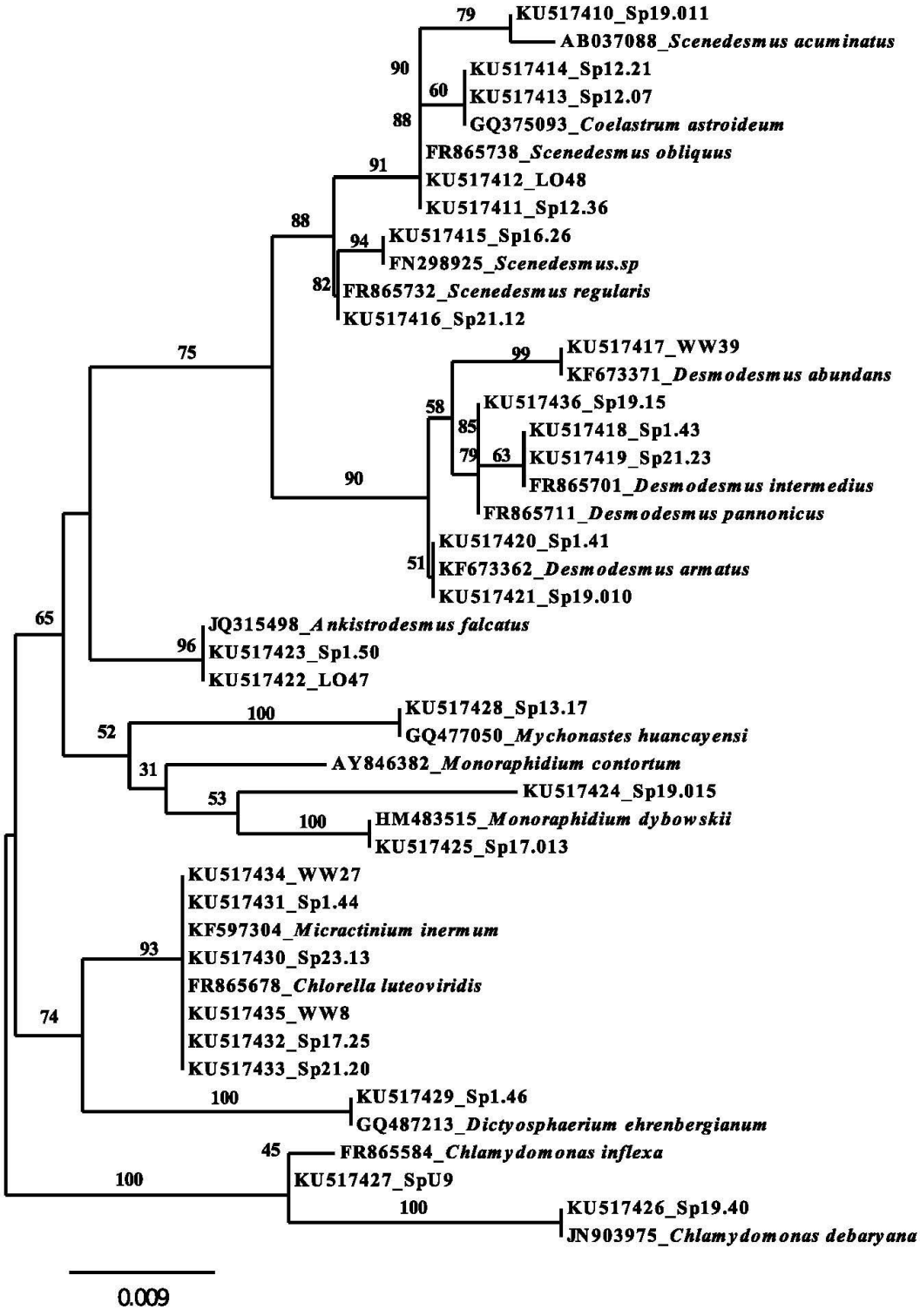
KU517410_Sp19.011
AB037088_Scenedesmus acuminatus
KU517414_Sp12.21
KU517413_Sp12.07
GQ375093_Coelastrum astroideum
FR865738_Scenedesmus obliquus
KU517412_LO48
KU517411_Sp12.36
KU517415_Sp16.26
FN298925_Scenedesmus.sp
FR865732_Scenedesmus regularis
KU517416_Sp21.12
KU517417_WW39
KF673371_Desmodesmus abundans
KU517436_Sp19.15
KU517418_Sp1.43
KU517419_Sp21.23
FR865701_Desmodesmus intermedius
FR865711_Desmodesmus pannonicus
KU517420_Sp1.41
KF673362_Desmodesmus armatus
KU517421_Sp19.010
JQ315498_Ankistrodesmus falcatus
KU517423_Sp1.50
KU517422_LO47
KU517428_Sp13.17
GQ477050_Mychonastes huancayensi
AY846382_Monoraphidium contortum
KU517424_Sp19.015
HM483515_Monoraphidium dybowskii
KU517425_Sp17.013
KU517434_WW27
KU517431_Sp1.44
KF597304_Micractinium inermum
KU517430_Sp23.13
FR865678_Chlorella luteoviridis
KU517435_WW8
KU517432_Sp17.25
KU517433_Sp21.20
KU517429_Sp1.46
GQ487213_Dictyosphaerium ehrenbergianum
FR865584_Chlamydomonas inflexa
KU517427_SpU9
KU517426_Sp19.40
JN903975_Chlamydomonas debaryana
0.009

Fig. 1

Figure 2(on next page)

FAME Profiles

FAME profiles for thirty-four axenic algal isolates based on the relative abundance of fatty acids detected by GC-MS.

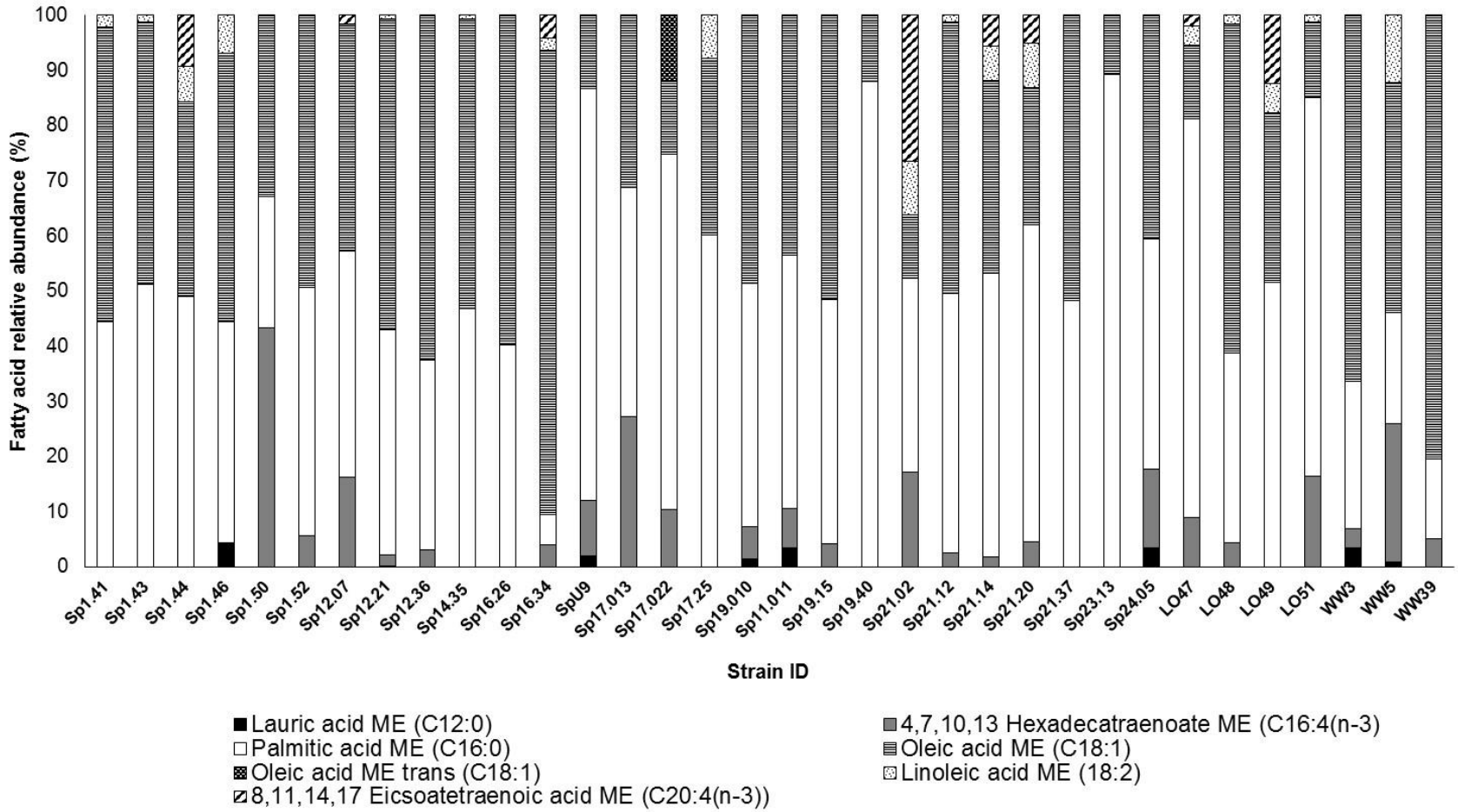
Fatty acid relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Sp1.41
Sp1.43
Sp1.44
Sp1.46
Sp1.50
Sp1.52
Sp12.07
Sp12.21
Sp12.36
Sp14.35
Sp16.26
Sp16.34
SpU9
Sp17.013
Sp17.022
Sp17.25
Sp19.010
Sp11.011
Sp19.15
Sp19.40
Sp21.02
Sp21.12
Sp21.14
Sp21.20
Sp21.37
Sp23.13
Sp24.05
LO47
LO48
LO49
LO51
WW3
WW5
WW39
Strain ID
Lauric acid ME (C12:0)
Palmitic acid ME (C16:0)
Oleic acid ME trans (C18:1)
8,11,14,17 Eicsoatetraenoic acid ME (C20:4(n-3))
4,7,10,13 Hexadecatraenoate ME (C16:4(n-3)
Oleic acid ME (C18:1)
Linoleic acid ME (18:2)

Fig. 2

Figure 3(on next page)

Comparison of FAME profiles among phylotytypes.

Comparison of FAME profiles among distinct phylotypes with 2 or more isolated strains using Bray-Curtis Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis. Strains of the same phylotype appear as the same colour in the dendrogram

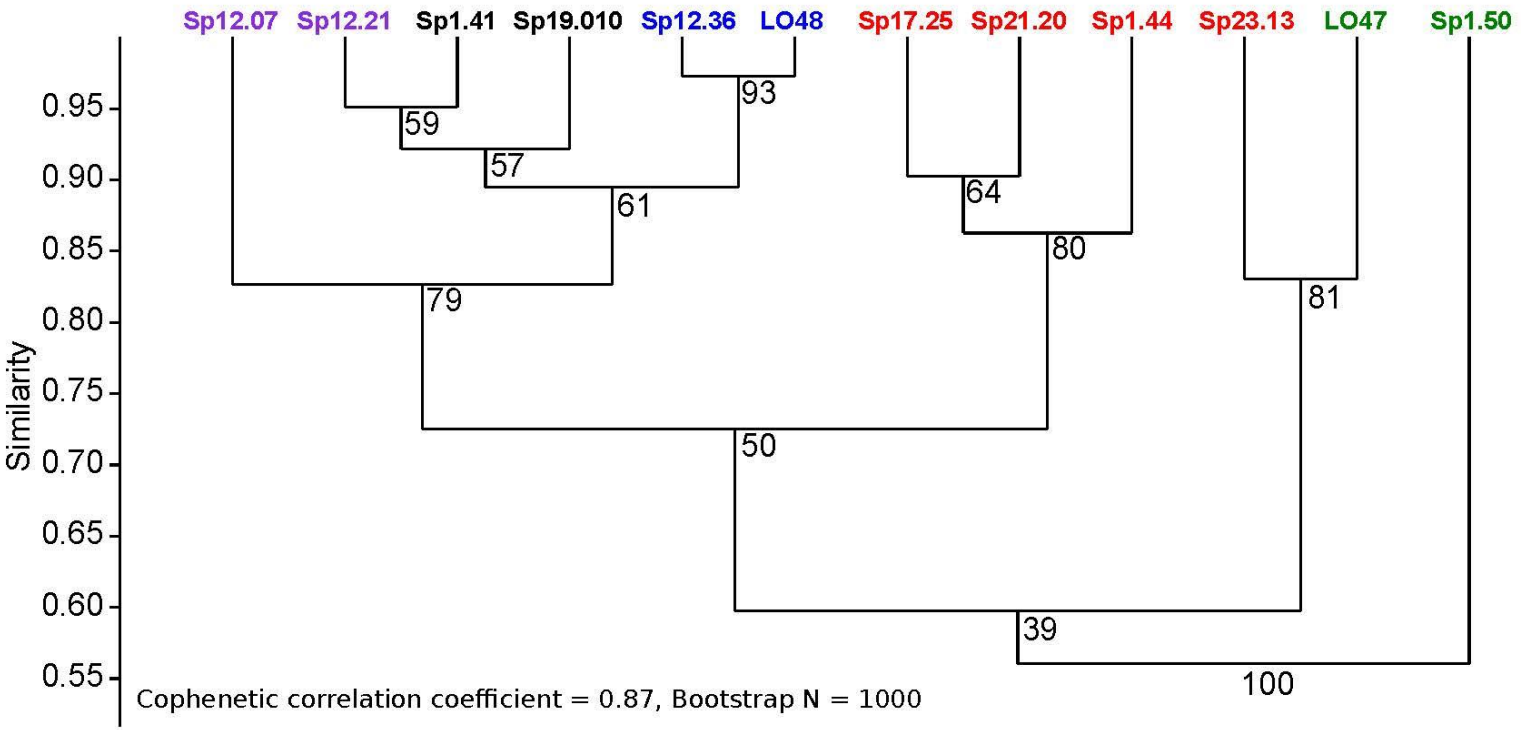
Sp12.07
Sp12.21
Sp1.41
Sp19.010
Sp12.36
LO48
Sp17.25
Sp21.20
Sp1.44
Sp23.13
LO47
Sp1.50
Similarity
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
93
59
57
61
64
80
79
81
50
39
100
Cophenetic correlation coefficient = 0.87, Bootstrap N = 1000

Fig. 3