Characterization of algae from urban stormwater and wastewater systems as candidates for biofuel feedstock

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Exploiting algae 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 algae 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 algae 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. 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 algae 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 algae 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 algae 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. 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.

Keywords: algae; fatty acids; FAME; 18SrRNA, copper tolerance, wastewater; biofuel

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, algae can be cultivated using saline, brackish or wastewater streams on non-arable land. Additionally, algal feedstock could 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 algae-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

may not be as critical as previously thought, because fast growth encourages high biomass productivity, and high biomass density can increase yield-per-harvest volume. Research to increase growth rates and lipid production has identified optimal temperatures, pH, photoperiods and nutrients for several species (Lv et al. 2010, Sforza et al. 2012, Adams et al. 2013). These parameters, however, are dissimilar among strains and require individual optimization.

Moving forward, it is recognized that bioprospecting for biofuel algae will require assessments of individual and mixed-cultures of strains. Additionally, ideal candidate strains would have inherent tolerance to sub-optimal growth conditions, since wastewater from a variety of sources may be the most economically and environmentally feasible growth medium. As a first step in assessing temperate-climate algae for their potential as biofuel feedstock, we aimed to isolate and culture strains from several engineered wastewater systems that included urban stormwater ponds and a municipal wastewater system. These environments were targeted because they are known to support algal communities, but also experience fluctuating conditions that include variation in contaminant loads and residence-time.

For example, we know that stormwater ponds typically have elevated metal concentrations (Campbell 1994; Marsalek and Marsalek 1997; Karlsson et al. 2010), including the ponds in our study (Vincent and Kirkwood 2014). It also has been well documented that municipal wastewaters have elevated levels of metals and other contaminants (Chambers et al. 1997; Gagnon and Saulnier 2003; Wang et al. 2005; Principi et al. 2006). Hence, these engineered environments were considered promising source habitats for algae with inherent tolerance to fluctuating conditions and contaminant loads, including metals. Overall, the 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

Strain Collection and 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 grown in 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). F2 vitamin mixture was added by syringe filter (Progene) to both media types after autoclaving. Isolation of unialgal strains was accomplished by methods outlined by Andersen and Kawachi (2005). Mixed-culture samples were serially diluted, 1 in 10 with sterile millipore water and spread plated on 1.5% agar plates. Visible colonies were pegged with sterile loop and re-cultured in original BG11 or CHU 10 media throughout the period of the study with re-culturing every three weeks. Isolated strains

92 were observed using an EVOS xl core inverted microscope at 400X to ensure unialgal status.
93 Tentative taxonomic strain identification was assigned based upon morphological descriptions in
94 Sheath and Wehr (2003).
95
96 *Growth Assays*
97 Growth assays were conducted under standardized conditions to determine growth rate
98 and generation time for all isolates. A 10-mL inoculum of exponentially-growing stock-culture
99 was aseptically introduced to 90-mL of sterile isolation media and grown in cotton-stoppered
100 flasks on a cool-white fluorescent plate-glass light table (20-22 ˚C, 12:12 dark/light cycle) and
101 shaken by hand once a day. Algal cell concentration was determined by microscopic
102 enumeration using a Brightline haemocytometer on a VistaVision compound light microscope at
103 100X magnification. The optical density of 1-mL samples was measured at 550nm every 24-hrs
104 for a seven-day period using the Genesys 10S UV/VIS Spectrometer. These data were used to
105 construct growth curves for each algal strain. Specific growth rate (μ), and generation time (T_h)
106 were calculated as per Guillard (1973) over a period of exponential growth.
107
108 *Antibiotic Treatment of Algal Isolates*
109 Great care was taken to attain axenic cultures using a modified antibiotic treatment
110 method adapted from Jones et al. (1973). Isolated strains were treated with a combination of
111 Penicillin G sodium salt and Streptomycin sulfate (Bioshop Canada) for an exposure period of 24
112 hours. Aliquots of the exposed strains were transferred to fresh media after 24 hours, followed by
113 pour plate preparation, 1% peptone and 1.5% agar, to evaluate bacterial contamination in
114 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 achieved or if antibiotics were found to inhibit algal growth. 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 reactions 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.

Phylogenetic tree reconstruction was performed on the Phylogeny.fr platform (Dereeper et al. 2008; Dereeper et al. 2010), which comprised a series of steps in the analysis. Sequences were aligned and configured with the highest accuracy using MUSCLE (v 3.8.31) (Edgar 2004). After alignment, ambiguous regions containing gaps or poorly aligned regions were removed with Gblocks (v0.91b) using the following parameters: minimum length of a block after gap cleaning=10; no gap positions were allowed; all segments with contiguous non-conserved positions bigger than 8 were rejected; and minimum number of sequences for flank position was 85% (Castresana 2000). The phylogenetic tree was reconstructed using the neighbour-joining method (Saitou and Nei 1987, Gascuel 1997) implemented in the BioNJ program with 1000

bootstrap replicates (Felsenstein 1985; Elias and Lagergren 2007). The analysis involved 45 nucleotide sequences with an average sequence length of 735 bp. Graphical representation and editing of the phylogenetic tree was performed with TreeDyn (v198.3) (Chevenet et al. 2006).

Fatty Acid Methyl Esters (FAME) Analysis

To avoid possible confounding effects of bacteria, only axenic isolates (thirty-four in total) were subjected to fatty acid analysis. Isolated strains were grown to stationary phase under previously described growth conditions and 10-mL subsamples were transferred to 14-mL glass tubes with lids and centrifuged at 16000 rpm (Sorval ST16, Thermo Scientific, USA) for five minutes. Pelleted biomass was frozen at -20°C for 48 hours followed by 24-48 hrs of lyopholization with a modulyoD freeze dryer (Thermo Scientific, USA). A direct method of transesterfication of algal fatty acids was performed as per O'fallon et al. (2007). A 100 ppm nonadecanoic acid methyl ester C19:0 (Sigma Aldrich, Canada) internal standard was added to 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.

Copper Toxicity Bioassays

Five isolates representing diverse taxonomic genera were further characterized to assess their inherent tolerance to copper, a particularly toxic metal to algae 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

207 described and data were normalized to percent-control. Growth-rate data were also used to
208 calculate percent inhibition:

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

209

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

211

212 **Results**

213 In total, forty-three algal strains were isolated from all habitat types. Tentative taxonomic
214 assignments were given to all isolates and, with the exception of one cyanobacterial strain
215 (*Microcystis sp.* Sp21.01), NCBI BLAST searches of partial 18S rRNA sequences were
216 performed (Table 1). All eukaryotic isolates are members of the algal division Chlorophyta,
217 which is a diverse group of green algae. Taxonomic assignment and BLAST matches were fairly
218 congruent, but there were several exceptions. All isolates were initially characterized for growth-
219 rate under standardized conditions (Table 1), and for all strains, exponential growth was most
220 commonly observed to last up to three days. Fastest growing strains came from stormwater
221 ponds and included *Scenedesmus sp* Sp19.011 and *Desmodesmus sp.* Sp19.15, which doubled
222 their biomass in just over 13 hours. In contrast, the slowest growing strains *Scenedesmus sp.*
223 Sp21.12 and *Chlorella sp.* Sp21.20 had generation times between 40-42 hours. Over 60 % of the
224 strains were found to have a generation time less than 24 hours (Table 1).

225 A subset of twenty-seven isolates with NS1sequences greater than 400 bp were used with
226 eighteen NCBI database-taxa to reconstruct an unrooted neighbour-joining phylogenetic tree
227 (Fig. 1). All strains included in the tree belong to the Chlorophyceae and Trebouxiophyceae
228 classes. Tree topology distinctly groups isolates into families and genera. In total, the tree
229 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.

A subset of thirty-four axenic isolates were subsequently assessed for their fatty-acid profiles (Fig. 2). Seven different fatty acids were detected among isolates and included 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 percentage (70-90%) of their total FAME lipid as palmitic acid. *Desmodesmus sp.* WW39 and *Scenedesmus sp.* Sp16.34 had oleic acid greater than 80% of their total FAME lipid profile. *Chlorococcum sp.* Sp17.022 was the only strain observed to have oleic acid C18:1 present in both cis- and trans- forms. One of the most unique profiles belongs to *Chlorella sp*. Sp21.02, which was found to have 53% of its lipids in polyunsaturated forms (Fig. 2). There was no apparent distinction of FAME profiles among strains isolated from different source-locations (Fig. 3).

To determine if there was any congruence among strains with respect to lipid composition and phylogenetic relatedness, the FAME profiles of strains sharing a phylotype with at least one other strain were compared using hierarchical cluster analysis (Fig. 4). The Bray-Curtis similarity dendrogram grouped strains of the same phylotype as being most similar based on their FAME profile. The cophenetic correlation coefficient of 0.87 indicates that the dendrogram does a good job of preserving the pairwise distances among strains. Bootstrap scores were mixed, but were moderate to strong for most nodes in the dendrogram. Strains *Scenedesmus*

253 *sp*. Sp12.36 and *Scenedesmus sp*. LO48 are the same phylotype and had the most similar FAME
254 profiles (>95% similarity). In contrast, *Ankistrodesmus sp*. LO47 and *Ankistrodesmus sp.* Sp1.50,
255 which are the same phylotype, were only about 55% similar based on their FAME profiles. Even
256 so, they were grouped as most similar among isolates in the cluster dendrogram (Fig. 4).
257 To assess the biofuel potential of the lipid content measured for each isolate, several
258 measures of biodiesel quality were determined using the methodologies and equations outlined in
259 Ramos et al. (2009) and Nascimento et al. (2013). ASTM Standard D675 for biodiesel quality
260 requires a minimum Cetane Number (CN) of 47 and a maximum Iodine Value (IV) of 120.
261 Twenty-eight isolates had a CN greater than 47, and thirty isolates did not exceed an IV of 120
262 (Table 2). In combination, twenty-eight isolates had acceptable CN and IV values according to
263 the ASTM standard. The Degree of Unsaturation among isolates was quite varied, ranging from
264 very low (10.6) for *Chlorella sp*. Sp23.13 to very high (120) for *Ankistrodesmus sp*. Sp1.50.
265 Long-chain saturation factor also varied among isolates, where isolate *Scenedesmus sp.* Sp16.34
266 had the lowest value (0.55) by an order of magnitude, and *Chlorella sp.* Sp23.13 had the highest
267 value (8.94). The Saponification Value was less varied among strains, where most had values
268 varying from 200-210. Strain *Chlamydomonas sp*. SpU9 had a notably high SV of 241. The
269 majority of isolates had negative-integer values for Cold Filter Plugging Point. *Scenedesmus sp*.
270 Sp16.34 in particular, had a CFPP as low as -15 °C. In contrast, Sp23.13 had a relatively high
271 CFPP at 12 °C.
272 To assess the inherent tolerance of algal isolates to the common wastewater contaminant
273 copper, five representative-strains from a variety of taxonomic groups were subjected to static
274 growth-response bioassays. Half of the strains tested showed a clear hormetic effect from copper
275 at 0.01 $mg \cdot L^{-1}$ (Fig. 5). Two of these strains (*Chlorella kesslerii* CPCC26 and *Scenedesmus sp.*

Sp11.30) continued to present a hormetic effect at 0.1 $mg \cdot L^{-1}$ copper, yet were greatly inhibited at subsequently higher copper concentrations. The cyanobacterial strain *Microcystis sp.* Sp21.01 was the least tolerant to copper, having complete growth inhibition at 0.32 $mg \cdot L^{-1}$ or higher. The two stormwater pond isolates *Monoraphidium sp.* Sp17.38 and *Selenastrum sp.* Sp14.35 had significantly higher (Student's t-test $p<0.05$) tolerance to 0.32 $mg \cdot L^{-1}$ copper than the reference strain *Chlorella kesslerii* CPCC26 (Fig. 4). The most tolerant strain was *Selenastrum sp*. Sp14.35, which had the highest growth rate at 1 $mg \cdot L^{-1}$ copper. No strains could grow at the highest test concentration 3.2 $mg \cdot L^{-1}$. At municipal-wastewater relevant concentrations of copper, all test strains, with the exception of *Microcystis sp.* Sp21.01, had minimal to no growth inhibition at the highest copper concentration 0.1 $mg \cdot L^{-1}$ (Table 3).

Discussion

All isolated algal strains (with the exception of one cyanobacterial isolate) belong to the Chlorophyta, a class of algae that includes taxa commonly used in algal biofuel research and application (Mata et al. 2010). In particular, *Chlorella sp*. and *Scenedesmus sp*. are the most commonly used for biofuel feedstock, and our study isolated several strains from each of these genera. Chlorophyte taxa such as the strains from our study are easy to culture, which in part, is likely why they are common in biofuel studies. Yet, these taxa have also been shown to be relatively good lipid producers (Mata et al. 2010), which increases their utility as feedstock in biodiesel production. As such, all source locations in this study, and in particular stormwater ponds and the municipal wastewater treatment plant, were fruitful habitats for biofuel strains.

Several discrepancies between taxonomic assignment and BLAST match (Table 1) was not entirely unexpected as this can be a common scenario. Though many chlorophyte species

299 exhibit type-features, such as spine and coenobium formation, culture condition will often affect
300 these characteristics used in identification. Morphological heterogeneity also makes algal
301 identification challenging, so it is possible that some strains were misidentified. In contrast, 18S
302 rRNA sequencing can be useful in distinguishing phylotypes of morphologically similar strains.
303 This was evident in the phylogenetic tree reconstruction, which provided a clear resolution to
304 generic assignment for isolates in the tree. In most instances, morphological similarities of clade
305 members were supported by genetic homology presented in the tree organization. BLAST
306 sequence searches for some of the more closely related *Scenedesmus, Desmodesmus* and
307 *Coleastrum* taxa often returned similar local alignment matches for query cover and percent
308 identity, making them difficult to confirm solely based on closest-match results. Again, the
309 phylogenetic tree topology provided useful clarification of misidentifications found in the
310 BLAST database. Sequence databases are known to have a small percentage (~20%) of their
311 sequence collection to be mislabelled or misidentified (Bridge et al, 2003, Vilgalys, 2003), and
312 algal systematics is currently in flux. As such, definitive taxonomic and phylogenetic assignment
313 for strains in this study will likely require further classification in the future.
314 Rapid growth rates are an important characteristic of biofuel feedstock, since fast-
315 growing strains would have increased yields in a shorter time-frame. According to Griffiths and
316 Harrison (2009), the average doubling time for green algae is 24 h, which corresponds to a
317 growth-rate (μ) = 0.69 d^{-1}. Of the forty-three isolates evaluated, the majority of strains were able
318 to double their biomass in 24-hours or less (Table 1). Twelve isolates from our study could grow
319 $\geq$ 1.0 d^{-1} (Table 1), which is considered to be a relatively fast growth-rate for microalgae in
320 general, and thus a desirable trait for biofuel-feedstock. Many of the fastest growing taxa were
321 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. 4). However, this finding deviates somewhat 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), 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 algae 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.

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 reflecting a ~50/50 saturated/unsaturated blend.

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

Although a select, but diverse group, of isolates were tested for copper tolerance, all chlorophyte strains exhibited high tolerance at wastewater-relevant concentrations (Table 3). Thus it is plausible that many chlorophyte isolates in our collection would likely have some inherent tolerance to copper if one extrapolates from the copper bioassay results. Others have also recognized the higher tolerance thresholds of chlorophyte algae to wastewater conditions, focusing primarily on *Chlorella* and *Scenedesmus* strains (Lau et al. 1995; Bhatnagar et al. 2010; Ruiz-Marin et al. 2010). Interestingly, the only non-chlorophyte strain in our study (*Microcystis*) was uniquely less tolerant to copper than the chlorophyte strains, even though it was also isolated from a stormwater pond. It is also interesting that two of the stormwater pond isolates *Monoraphidium sp.* Sp17.38 and *Selenastrum sp.* Sp14.35 were notably tolerant to copper at concentrations orders of magnitude higher than their source stormwater ponds (Table 3). Therefore, these strains in particular may be ideal candidates for industrial-wastewater feedstock since they are relatively fast growers (Table 1), have ideal fatty-acid profiles (Fig. 2 and Table 2), and have inherent tolerance to the algicidal-metal copper (Fig. 5).

Bioprospecting of algal strains for use in biofuel production is a relatively new trend. Though our study is the first to target biofuel bioprospecting efforts in stormwater ponds and a municipal wastewater system, the dominant taxa isolated, including *Chlorella, Scenedesmus, and Desmodesmus,* are cosmopolitan in freshwater environments. Strains identified as the species *Scenedesmus obliquuss* and *Chlorella leuteoiviridis* in blast matches were prevalent in both stormwater ponds and the wastewater treatment facility, and they typically out-competed other species during cultivation. Zhou et al. (2011) found similar results in isolations of microalgae from wastewater sites in the United States, isolating several species of *Chlorella*. Past efforts to isolate algae from various habitats including wastewater systems was mainly for wastewater

remediation. *Chlorella* and *Scenedesmus* are common taxa isolated for this purpose, especially for nutrient removal (Tam and Wong 1989; Tam and Wong; 1990, Gonzalez et al. 1997; Woertz et al. 2009; Chinnasamy et al. 2010). Nutrient removal by algae cultivated in wastewater would provide a value-added benefit during biofuel-feedstock production.

While the results presented here are only a first step in assessing algal strains from wastewater habitats as biofuel-feedstock candidates, the ultimate goal is to establish and select strains most tolerant to municipal and/or industrial wastewater conditions. The use of wastewater to support algal biomass production is not a new approach, but it is for biofuel feedstock production. Several studies have all concluded that there is great potential for algal biofuel production using wastewater (Pittman et al. 2011; Bhatnagar et al. 2011; Christenson and Sims 2011). Yang et al. (2011) looked at the total water and nutrient requirements of algae-based biofuels and found that biofuels sourced from wastewater decreased total water requirements by 90% in comparison to freshwater sourcing without recycling. What has not been investigated explicitly is the viability of using wastewater on a continuous basis in a pond or photobioreactor system growing single or mixed strains of algae. Broad variations can occur in wastewater quality, in addition to fluctuations in toxic-metal constituents (Cole et al. 1989; Ahuwalia et al. 2007). In the case of municipal wastewater, the nutrient and contaminant content can vary daily depending on the source-population. As such, future studies of biofuel algae, including promising isolates from this study, would need to be assessed for their tolerance and resilience to 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, and tentative taxonomic assignment based on morphological features in culture. 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. Taxa with the closest sequence matches (>90% similarity) from the NCBI BLAST are included for each isolate. 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.

Table 1. Summary list of isolated strains and their associated isolation medium, source location, and tentative taxonomic assignment based on morphological features in culture. 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. Taxa with the closest sequence matches (>90% similarity) from the NCBI BLAST are included for each isolate. 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.

Strain ID	Isolating Medium	Source Location	Taxonomic Assignment	Closest BLAST Match	Tot. Seq. Size (bp)	Growth Rate (d^{-1})	Generation Time (hrs)
Sp1.41	CHU10	SWP 1	*Scenedesmus sp.*	*Desmodesmus armatus KF673362*	1160	1.14	14.6
Sp1.43	CHU10	SWP 1	*Desmodesmus sp.*	*Desmodesmus intermedius* FR865701	1253	0.74	21.1
Sp1.44	CHU10	SWP 1	*Chlorella sp.*	*Chlorella sp.* KP262476	1580	0.75	22.2
Sp1.46	CHU10	SWP 1	*Dictyosophaerium sp.*	*Dictyosphaerium ehrenbergianum* GQ487213	474	0.69	24.0
Sp1.50	CHU10	SWP 1	*Ankistrodesmus sp.*	*Ankistrodesmus gracilis. AB917098*	1618	1.15	14.5
Sp1.52	CHU10	SWP 1	*Chlorella sp.*	*Mychonastes rotundus* GQ477053	343	0.97	17.2
Sp11.30	CHU10	SWP 11	*Scenedesmus sp.*	*Scenedesmus obliquus* FN298925	911	0.51	32.6
Sp12.07	BG11	SWP 12	*Chlorella sp.*	*Coelastrum astroideum* GQ375093	1518	0.55	30.2
Sp12.21	CHU10	SWP 12	*Chlorella sp.*	*Coelastrum astroideum* GQ375093	850	1.04	16.1
Sp12.36	CHU10	SWP 12	*Scenedesmus sp.*	*Acutodesmus obliquus AB917101*	1614	0.90	18.6
Sp13.17	CHU10	SWP 13	*Chlorella sp.*	*Mychonastes huancayensi* GQ477050	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	1431	0.90	18.6
Sp16.34	CHU10	SWP 16	*Scenedesmus sp.*	*Scenedesmus obliquus* KJ676128	714	1.10	15.1
SpU.9*	CHU10	SWP 16	*Chlamydomonas sp.*	*Chlamydomonas inflexa* FR865584	1143	0.59	28.1

Sp17.013	BG11	SWP 17	*Monoraphidium sp.*	*Monoraphidium convolutum* HM483515	1309	0.92	18.1
Sp17.022	BG11	SWP 17	*Chlorococcum sp.*	*Chlorococcum ellipsoideum* U70586	423	0.65	25.6
Sp17.25	CHU10	SWP 17	*Chlorella sp.*	*Chlorella luteoviridis* FR865678	1482	0.98	17.0
Sp17.38	CHU10	SWP 17	*Monoraphidium sp.*	*Monoraphiridium sp.* JN187941	873	1.12	14.9
Sp19.010	BG11	SWP 19	*Desmodesmus sp.*	*Desmodesmus communis* KF864475	1178	0.64	26.0
Sp19.011	BG11	SWP 19	*Scenedesmus sp.*	*Scenedesmus acuminatus AB037088*	1073	1.24	13.5
Sp19.015	CHU10	SWP 19	*Monoraphidium sp*	*Monoraphiridium contortum* AY846382	588	0.72	23.1
Sp19.15	CHU10	SWP 19	*Desmodesmus sp.*	*Desmodesmus pannonicus* FR865712	821	1.22	13.7
Sp19.40	CHU10	SWP 19	*Chlamydomonas sp.*	*Chlamydomonas debaryana* JN903975	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	718	0.80	20.7
Sp21.12	CHU10	SWP 21	*Scenedesmus sp.*	*Scenedesmus sp.* FR865732	1483	0.40	41.8
Sp21.14	CHU10	SWP 21	*Chlorella sp.*	*Chlorella luteoviridis* FR865678	449	0.75	22.1
Sp21.20	CHU10	SWP 21	*Chlorella sp.*	*Chlorella luteoviridis* FR865678	1336	0.42	39.8
Sp21.37	CHU10	SWP 21	*Ankistrodesmus sp.*	*Monoraphidium contortum* KM067465	626	1.10	15.1
Sp21.23	CHU10	SWP 21	*Desmodesmus sp.*	*Desmodesmus intermedius* FR865701	1245	1.07	15.6
Sp23.13	CHU10	RP 23	*Chlorella sp.*	*Micractinium inermum* KF597304	1005	0.55	30.3
Sp24.1	CHU10	RP 24	*Scenedesmussp.*	*Scenedesmus sp.* FN298925	257	0.55	30.3
Sp24.05	BG11	RP 24	*Desmodesmus sp.*	*Desmodesmus pannonicus* FR865712	1158	0.50	33.2
LO47	CHU10	Lake ON	*Ankistrodesmus sp.*	*Monoraphidium griffthii.* AY846383	1454	0.74	22.5
LO48	CHU10	Lake ON	*Scenedesmus sp.*	*Scenedesmus obliquus* FR865738	1119	1.00	16.7
LO49	CHU10	Lake ON	*Chlorella sp.*	*Micractinium sp. AB9187105*	350	1.08	22.2
LO51	CHU10	Lake ON	*Chlorella sp.*	*Chlorella luteoviridis. FR865678*	585	0.58	29.1
WW3	CHU10	WWTP	*Chlorella sp.*	*Coelastrum microporum* JQ315528	497	0.63	25.3
WW5	CHU10	WWTP	*Scenedesmus sp.*	*Acutodesmus obliquus* KF144164	597	0.58	28.7
WW8	CHU10	WWTP	*Chlorella sp.*	*Chlorella luteoviridis.* FR865678	761	0.80	20.7
WW27	CHU 10	WWTP	*Chlorella sp.*	*Chlorella luteoviridis* FR865678	1304	0.77	21.7
WW39	CHU10	WWTP	*Desmodesmus sp.*	*Desmodesmus intermedius* KF673371	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

7
8

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)	
Strain ID	**Source Location 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.

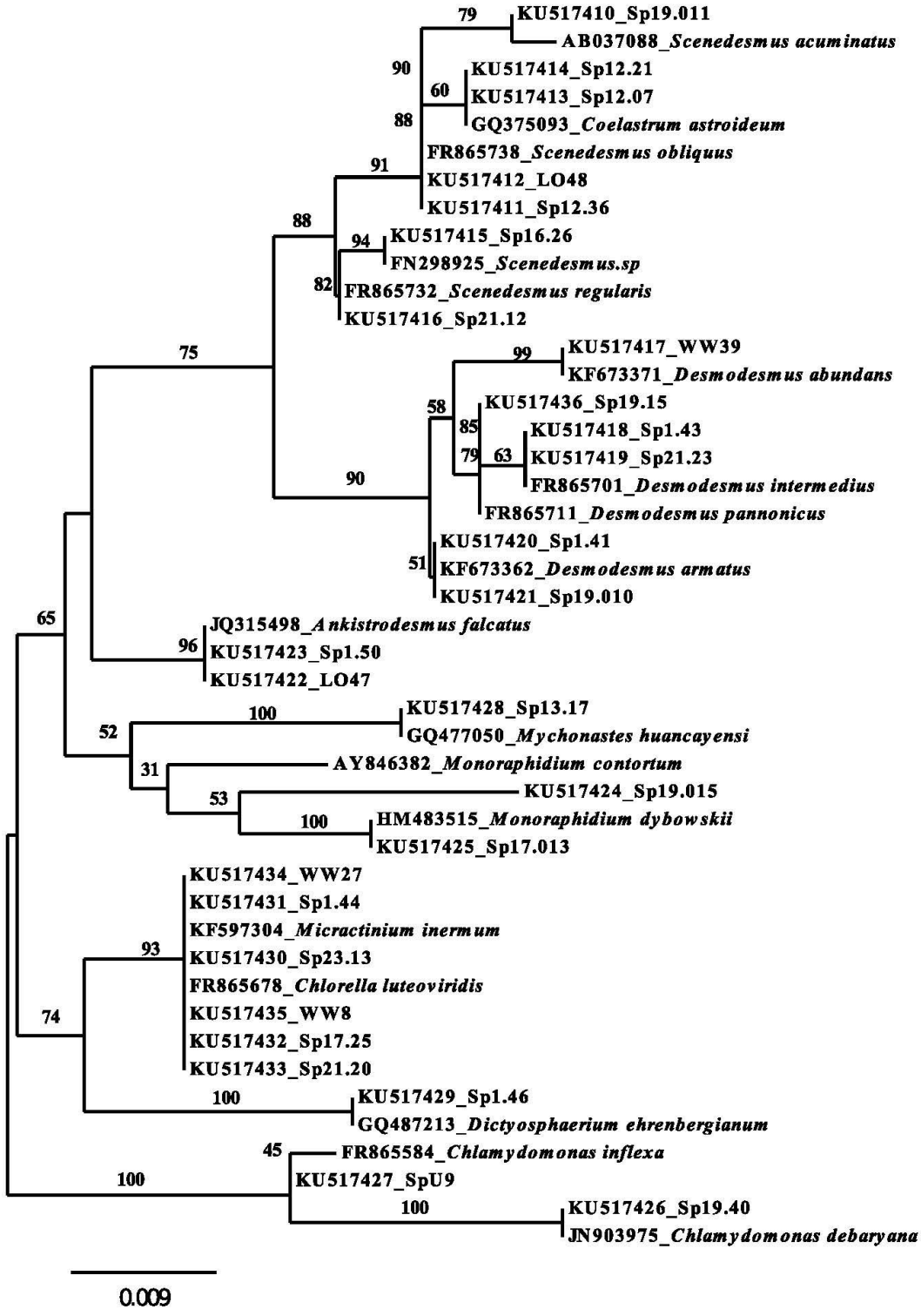
KU517410_Sp19.011
AB037088_Scenedesmus acuminatus
KU517414_Sp12.21
KU517413_Sp12.07
GQ375093_Coelastrum astroideum
FR865738_Scenedesmus obliquus
KU517412_LO48
KU517411_Sp12.36
KU517415_Sp16.26
FN298925_Scenedesmus.sp
FR865732_Scenedesmus regularis
KU517416_Sp21.12
KU517417_WW39
KF673371_Desmodesmus abundans
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.

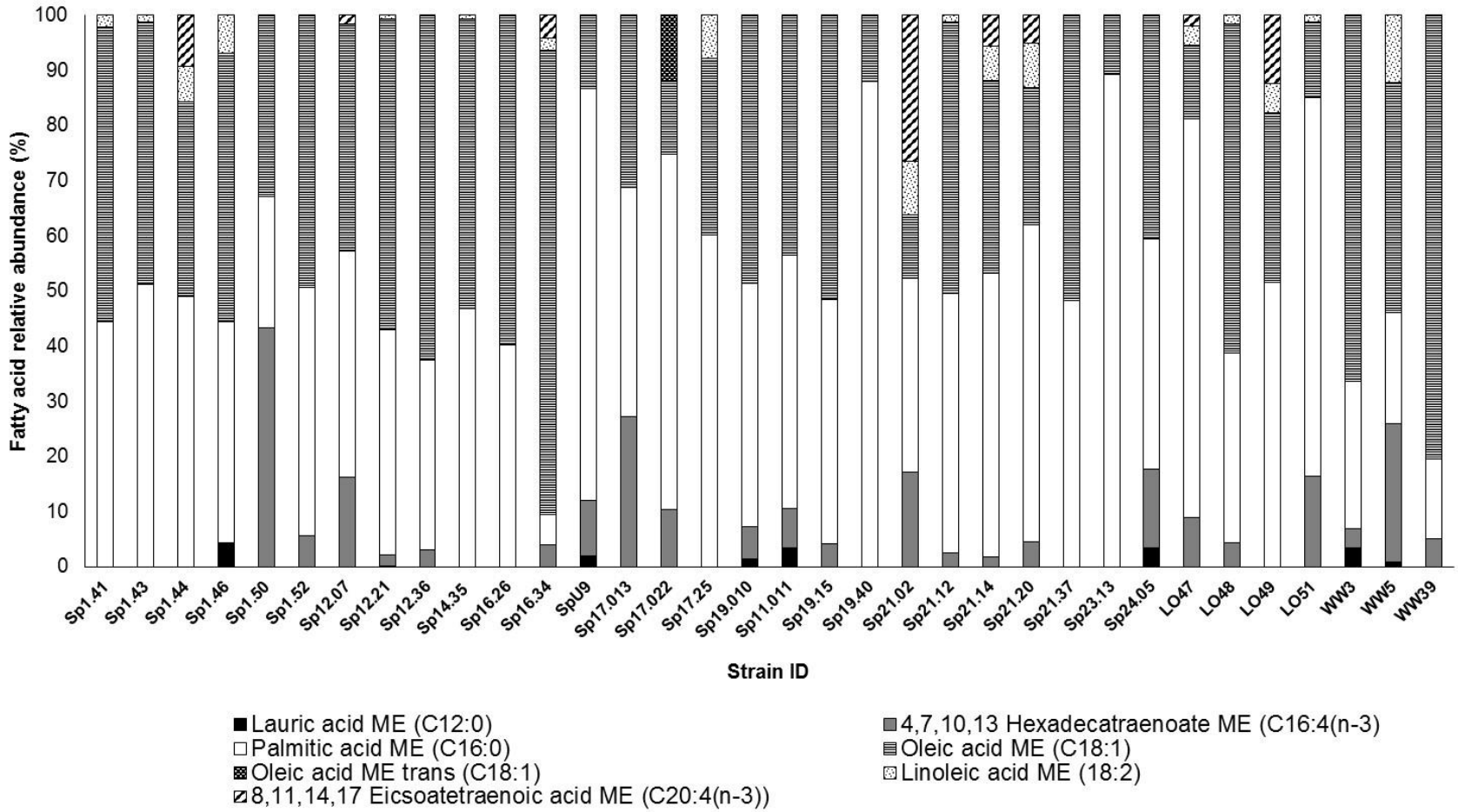
Fatty acid relative abundance (%)
100
90
80
70
60
50
40
30
20
10
0
Sp1.41
Sp1.43
Sp1.44
Sp1.46
Sp1.50
Sp1.52
Sp12.07
Sp12.21
Sp12.36
Sp14.35
Sp16.26
Sp16.34
SpU9
Sp17.013
Sp17.022
Sp17.25
Sp19.010
Sp11.011
Sp19.15
Sp19.40
Sp21.02
Sp21.12
Sp21.14
Sp21.20
Sp21.37
Sp23.13
Sp24.05
LO47
LO48
LO49
LO51
WW3
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)

Principal component analyses of fatty acid profiles

Principal component analyses of fatty acid profiles for thirty-four algal strains labelled by source location: Black circle = Stormwater Pond, Orange square = natural reference site, Red Diamond = Municipal Wastewater Treatment Plant. Vector lines represent individual fatty acids.

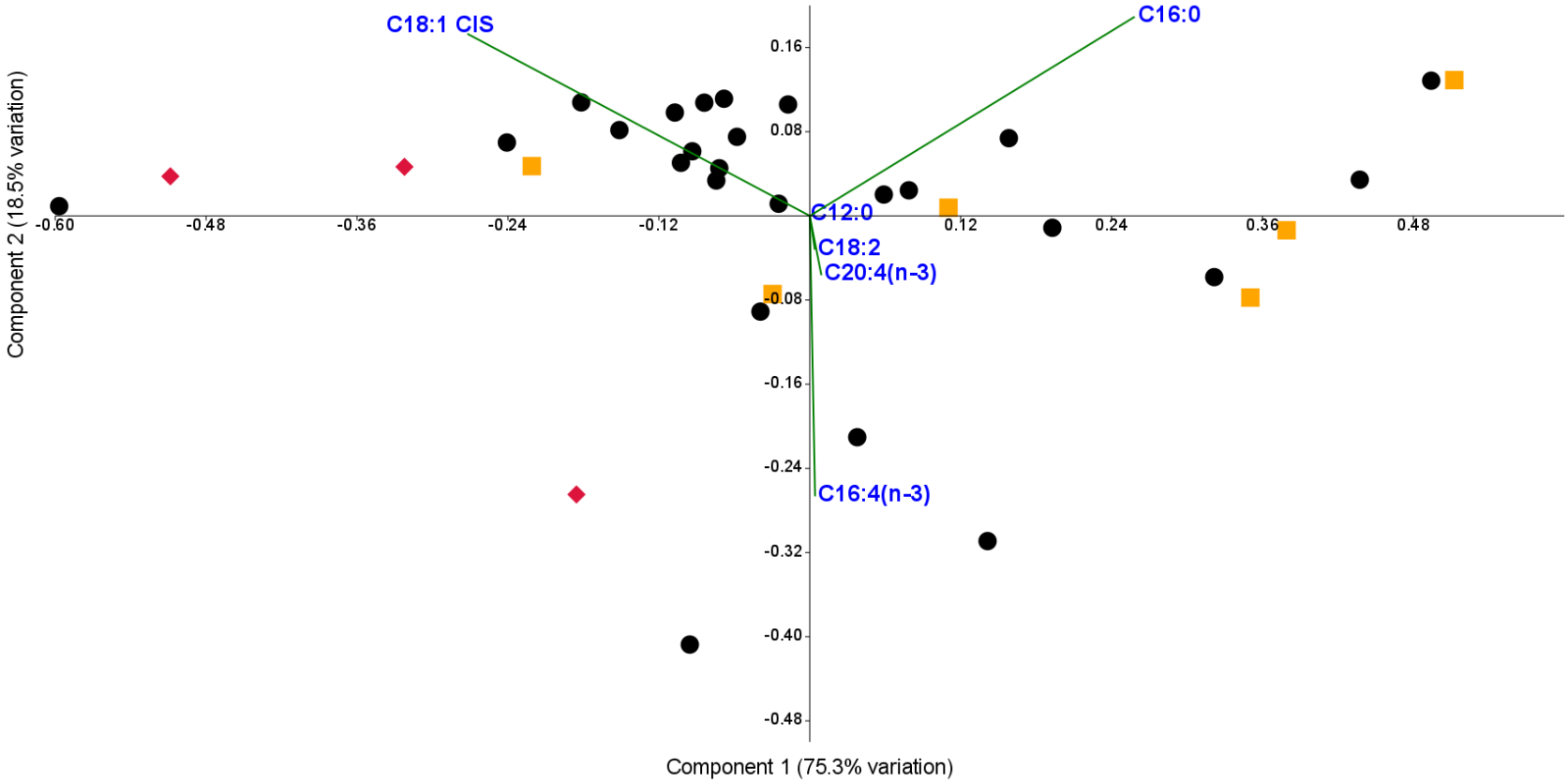
C18:1 CIS
C16:0
C12:0
C18:2
C20:4(n-3)
C16:4(n-3)
0.16
0.08
-0.08
-0.16
-0.24
-0.32
-0.40
-0.48
-0.60
-0.48
-0.36
-0.24
-0.12
0.12
0.24
0.36
0.48
Component 2 (18.5% variation)
Component 1 (75.3% variation)

Fig. 3

Figure 4(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

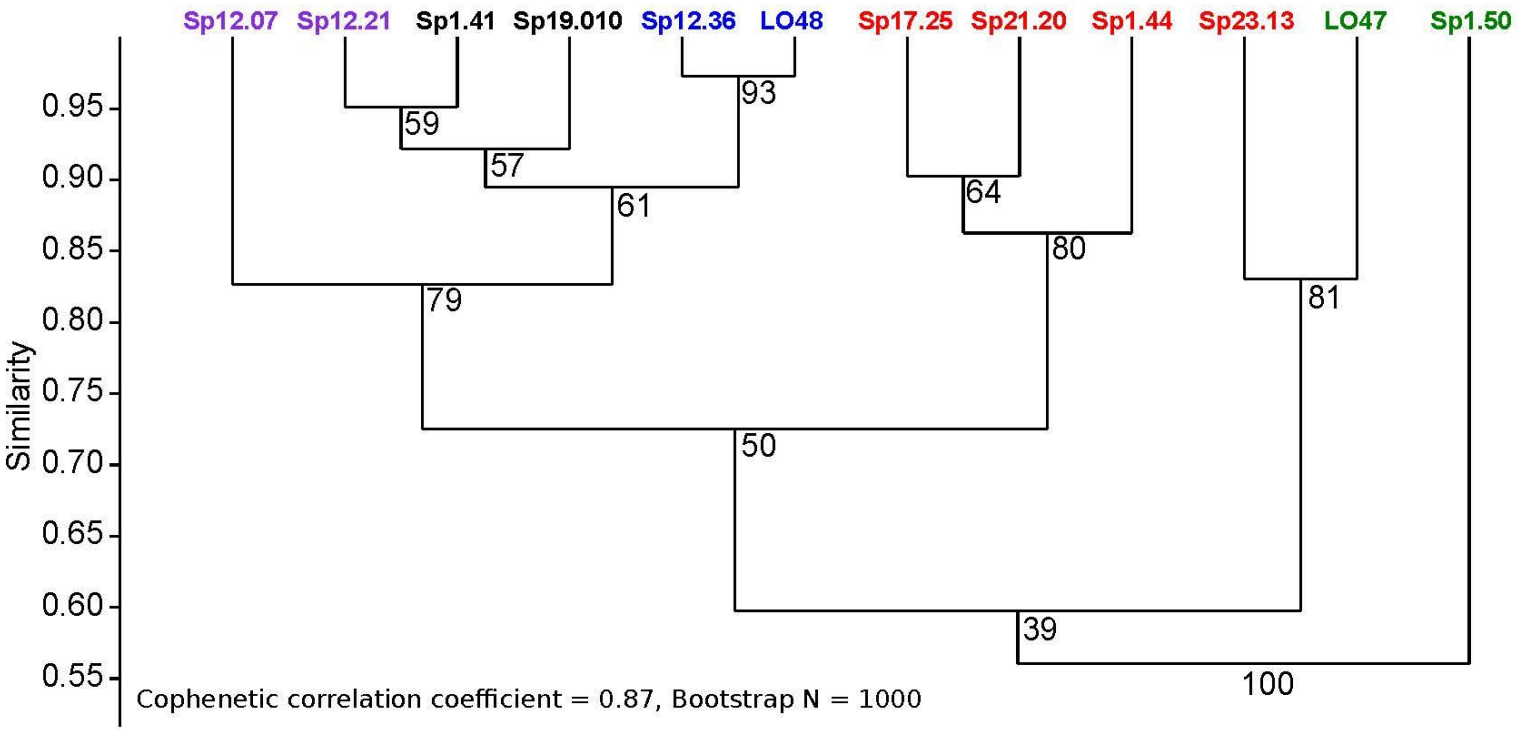
Sp12.07
Sp12.21
Sp1.41
Sp19.010
Sp12.36
LO48
Sp17.25
Sp21.20
Sp1.44
Sp23.13
LO47
Sp1.50
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
Similarity
59
57
93
61
64
80
79
81
50
39
100
Cophenetic correlation coefficient = 0.87, Bootstrap N = 1000

Fig. 4

Figure 5(on next page)

Comparison of copper tolerance.

Comparison of copper tolerance among select algal isolates from stormwater ponds and a culture collection reference-strain (CPCC 26) commonly used in metal bioassays. Algal growth rate was the response variable normalized to percent control of each strain.

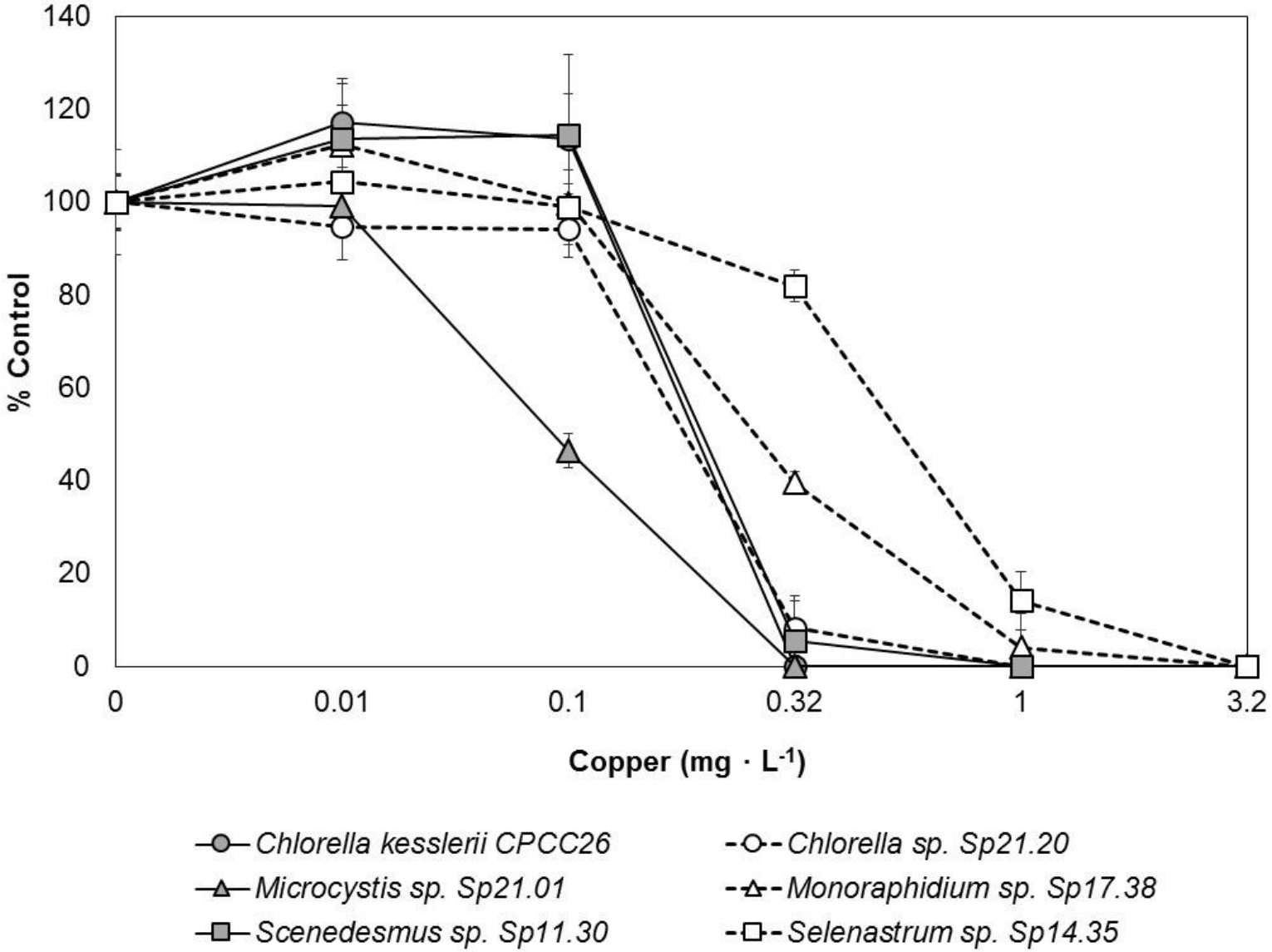
140
120
100
80
60
40
20
0
% Control
0
0.01
0.1
0.32
1
3.2
Copper (mg · L-1)
Chlorella kesslerii CPCC26
Chlorella sp. Sp21.20
Microcystis sp. Sp21.01
Monoraphidium sp. Sp17.38
Scenedesmus sp. Sp11.30
Selenastrum sp. Sp14.35

Fig. 5