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Short-term deleterious effects of standard isolation and cultivation methods on new tropical freshwater microalgae strains

M. Magdalena Aray-Andrade[1,2], Miguel I. Uyaguari-Diaz[3,4], J. Rafael Bermúdez[1,5]*

[1] Plankton Laboratory, Facultad de Ingeniería Marítima, Ciencias Biológicas, Oceánicas y Recursos Naturales. Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil, Ecuador.

[2] Joint postgraduate VLIR NETWORK Master Program in Applied Biosciences, Biodiscovery, Escuela Superior Politécnica del Litoral, ESPOL, Guayaquil, Ecuador.

[3] British Columbia Centre for Disease Control Public Health Laboratory, Vancouver, British Columbia, Canada V5Z 4R4.

[4] Department of Pathology and Laboratory Medicine, The University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4

[5] Galapagos Marine Research and Exploration, GMaRE. Joint ESPOL-CDF program, Charles Darwin Research Station, Galapagos Islands, Ecuador.

*Contact Author:

J. Rafael Bermúdez

Km 30, Vía Perimetral 5, Guayaquil

jrbermud@espol.edu.ec

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Keywords: cryopreservation, lipids, growth rate, microalgae, antibiotics

Summary

Algae with potential biotechnological applications in different industries are commonly isolate from the environment in order to obtain pure (axenic) stocks that can be safely stored for long periods of time. To obtain axenic cultures, antibiotics are frequently used, and cryopreservation is applied to keep standing stocks. However, many of these now standard methods were developed using strains coming from pristine-to-low intervened environments and cold-to-temperate regions. Thus, it is still not well understand the potential effect of said methods on the life cycle and biochemical profile of algae isolates from hiper-eutrophic and constant high-temperature tropical regions, which could potentially render them unsuitable for their intended biotechnological application. In this study, we conducted a genetic characterization (18S rRNA) and evaluated the effect of purification (use of the antibiotic chloramphenicol, CPA) and cryopreservation (dimethyl sulfoxide (DMSO)-sucrose mix and Glycerol) on the growth rate and lipid content of four new tropical freshwater algal isolates: *Chorella* sp. M2, *Chlorella* sp. M6, *Scenedesmus* sp. R3, and *Coelastrella* sp. A2, from the Ecuadorian coast. The genetic characterization showed a clear discrimination between strains. All strains cultured with CPA had a lower growth rate. After cryopreservation *Coelastrella* sp. A2 did not grow with both methods; *Chorella* sp. M2, *Chlorella* sp. M6 and *Scenedesmus* sp. R3 presented no significant difference in growth rate between the cryopreservants. A significantly higher lipid content was observed in biomass cryopreserved with glycerol in relation to DMSO-sucrose, with *Chorella* sp. M2 and *Chlorella* sp. M6 having twice as much in the first treatment. The results highlight the relevance of choosing an appropriate method for storage as the materials used can affect the

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biological performance of different tropical species, although is still to determine if the effects observed here are long lasting in subsequent cultures of these algae.

Introduction

The cultivation of wild microalgae under defined/controlled conditions has received considerable interest due to their potential as a source of various compounds with biotechnological and commercial significance in nutraceutical, cosmetic and pharmaceutical industries (Gellenbeck, 2011; Encarnação *et al*., 2015; Rodrigues *et al*., 2015; Dogaris *et al*., 2015; Maurya *et al*., 2016). Some microalgae are essential raw materials in biofuel production because of their ability to accumulate a high lipid content. Microalgae such as *Chlorococcum* sp. is a fast growing specie and its lipid content can contribute up to 30.55 ±2.65% in dried biomass (Mahapatra & Ramachandra, 2013); some *Chlorella* species are known to produce lipid ranged from 10–39% in dried biomass (Chiu *et al*., 2015), showing a huge potential as lipid sources in biotechnology. Nowadays the production of microalgal biomass has been upscaled to large outdoors cultivation systems for the production of biodiesel. However, high temperatures can severely affect the productivity of said systems (Béchet *et al*., 2013). For this reason, it has become important the isolation and characterization of high temperature-tolerant microalgal species in order to mitigate said effects, as has been demonstrated with the temperature-tolerant strain *Chlorella sorokiniana* (Béchet *et al*., 2013).

An additional advantage of algal biomass production is the potential use of wastewater as culture media for the production of middle and high-value by-products. The use of wastewater is beneficial as it reduces the use of freshwater, decrease the cost of nutrient addition, and helps in the removal of nitrogen and phosphorus which otherwise would end in the environment (Chiu *et al*., 2015), and have been show that several microalgae have high bioremediation potential as

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Chlorella and *Scenedesmus* (Silkina *et al*., 2017). However, wastewater has an elevated amounts of nitrogen, phosphorus and microbes, which could affect algal productivity (Mara, 2004; Mahapatra & Ramachandra, 2013) Therefore, an efficient strategy to mitigate potential negative effects of the use of wastewater as culture media is the isolation of wild algal strains that proliferate in it (Mahapatra & Ramachandra, 2013). However, to assess the biotechnological potential and applications of microalgal species, the first step is to isolate them from the environment and to obtain pure (axenic) stocks that can be safely stored for long periods of time, and then can be used as stock for future cultures. Given that an elevated bacterial contamination can be expected during the isolation of fresh strains from wastewater (Mara, 2004), antibiotics such as florfenicol, streptomycin, furazolidone and specially chloramphenicol (CAP) are often used to eliminate bacteria in the culture media (Campa-Córdova *et al*., 2006; Lai *et al*., 2009), this last is highly effective because it inhibits a variety of aerobic and anaerobic microorganism. Nevertheless, an intensive use of antibiotics in households, agriculture and aquaculture have had adverse ecological effects, including the development of resistant bacterial populations (Lai *et al*., 2009), which may require a higher concentration of antibiotics to achieve its elimination from isolated strains. However, it has been reported that antibiotics can negatively affect the growth of certain algal species such as *Chlorella pyrenoidosa*, *Isochrysis galbana* and *Tetraselmis chui* (Lai *et al*., 2009) cultured with CAP.

Cryopreservation of microalgae stocks for long term storage is important as it eliminates the requirement of keeping continuous batches of growing strains (Bui *et al*., 2013). Although the mechanisms underlying cryopreservation are understood in general, the impact of these are not always elaborated (Bui *et al*., 2013) and it is not well understood their impact in potentially

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difficult-to-freeze algae, specially that from tropical environments, as this species have never experienced cold or desiccation, much less freezing in their life cycles (Fuller, Lane & Benson, 2004).

In general terms, the issues of cryopreservation is the forming ice crystals within the cellular cytoplasm during the freezing process, which disrupts the cell membrane (Bui *et al*., 2013). To reduce such effect, several cryopreservants can be added to the culture media to protect eukaryotic cells from damage caused by freezing. For example Dimethyl sulfoxide (DMSO) and methanol are cell wall-permeable cryoprotectants that rapidly enter the cell; glycerol is also permeable, but enters more slowly; while sucrose does not cross the cell membrane and thus its protection is extracellular (Hubálek, 2003; Bui *et al*., 2013). Furthermore, the cryopreservation of algae can change their original biochemical profile through the alteration of their genetic structure and/or metabolic pathways (Müller *et al*., 2007), making them unsuitable for their intended biotechnological application when they were originally isolated.

A crucial step for the potential use of tropical algae as a biotechnological resource is to determine the effects of cultivation process on their biochemical profiles and productivity rates. The aim of the present study was to evaluate the effect of traditional isolation and cultivation process used to obtain axenic culture (treatment with chloramphenicol) and their cryopreservation (DMSO-sucrose and glycerol) on the growth rate and lipid content of four fresh algae isolates from wastewater in the tropical Ecuadorian coast region.

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Materials and Methods

Isolation, identification and preparation of stocks

Microalgae strains were isolated from green standing water bodies (20 ml) in different standing ponds around the urban areas of Guayaquil, Ecuador. From each water sample collected, serial 1:10 dilutions were made in 1.5 ml eppendorf tubes using Guillard's f/2 medium(Guillard & Ryther, 1962) as culture media.

The strains, identified by morphological differentiation were: *Chorella* sp. M2, *Chlorella* sp. M6, *Scenedesmus* sp. R3. and *Coelastrella* sp. A2, following the method of Wehr & Sheath (2003).

The isolated and identified strains were cultivated in batch cultures with Guillard's f/2 medium, under controlled laboratory conditions (culture cabins at 30°C and a 12/12 hours photoperiod), to increase the biomass of each strain prior to the beginning of the experiments.

DNA extraction, polymerase chain reaction, cloning, and sequencing

Before extraction and to facilitate disruption of algal cells, eight freeze-thaw cycles were conducted followed by overnight digestion using 20 µl of 20 mg/ml of proteinase K (Qiagen Sciences, Germantown, MD). DNA was extracted using the UltraClean Soil DNA Kit (MoBio, Carlsbad, CA) as per the manufacturer's instructions. DNA concentration and purity was assessed with Qubit dsDNA high sensitivity in a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA) and NanoDrop spectrophotometer (NanoDrop technologies, Inc., Wilmington, DE), respectively. Specific primers targeting V1–V3 regions of the 18S rRNA gene were selected from previous studies (Amann, Krumholz & Stahl, 1990; Zhu *et al*., 2005) (Amann, Krumholz & Stahl, 1990; Zhu *et al*., 2005). Each 50 µl of PCR reaction consisted of 1.5-mM $MgCl2$, 0.2-mM nucleotides, 400 mM of each primer, 1.25 U of Hot Start Polymerase (Promega

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Corporation, Fitchburg, WI), and 1 µl of template DNA. The thermal cycling conditions consisted of 94 °C × 5 min, 35 cycles of 30 s at 94 °C, 60 s at 55 °C, and 90 s at 72 °C, and a final cycle of 10 min at 72 °C. PCR amplicons were examined in a 1.5% agarose/0.5X TBE gel stained with 1X GelRed (Biotium, Inc., Hayward, CA), and purified with a QIAQuick PCR Purification Kit (Qiagen Sciences, Maryland, MD) according to the manufacturer's instructions. Amplicons then were ligated into pCR2.1-TOPO cloning vectors (Invitrogen, Carlsbad, CA), and transformed into One Shot E. coli DH5 α-T1R competent cells following the manufacturer's protocol. At least 4 transformants per sample were screened for inserts by following manufacturer's recommendations. Positive transformants were then grown overnight at 37 °C in lysogeny broth with 50 µg ml-1 of kanamycin. Plasmids were extracted using PureLink Quick Plasmid Miniprep Kit (Life Technologies, Carlsbad, CA). Further quantitation assessment used a dsDNA high sensitivity kit in a Qubit 3.0 fluorometer (Life Technologies, Carlsbad, CA). Plasmids were end-sequenced in an ABI 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA) with M13 forward primer (-20) (5'-GTAAAACGACGGCCAG-3') and M13 reverse primer (5'- CAGGAAACAGCTATGACC-3') using BigDye Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Warrington, UK). The resultant set of DNA sequences were blasted against the SILVA 18S rRNA database (Pruesse *et al*., 2007).

Phylogenetic tree construction

To further define the sequences in this study, an unweighted pair group method with arithmetic mean (UPGMA) tree was constructed using BLASTN matches and other characterized 18S rRNA gene algal sequences using Geneious 9.1.8 software (Kearse *et al*., 2012). The robustness of the tree was tested using bootstrap analysis (1,000 times)

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Axenic Culture

To obtain axenic culture, chloramphenicol was added to Guillard's f/2 medium to a final concentration of 50 mg L^{-1}, as recommended by Lai *et al.* (2009). This concentration was used on account of the strains evaluated are wild microalgae, isolated around the market places in Guayaquil in stagnant water ponds, exposed to direct solar radiation, and expected to have an elevated nutrient content and bacterial load). The microalgae strains were cultivated in triplicates during 6 days at 30°C and photoperiod 12:12. For each strain, a negative control without added antibiotic was cultivated under the same conditions as the treated group. The growth rate of each culture was determined by microscopy counts using a Neubauer chamber (IOC, UNESCO, 2010) and expressed as cell ml^{-1}.

Cryopreservation Test

The test of DMSO-sucrose mix and glycerol treatments on each strain isolated was performed in triplicates. Cultures of *Chorella* sp. M2, *Chlorella* sp. M6 and *Scenedesmus* sp. R3, which had a concentrations in the range of 2 - 9 × 10^7 ml^{-1} cells were selected to evaluate the effect of cryopreservants, as recommended by Bui (2013). From each culture 1 ml was transferred to two 1.8 ml cryovials; in one vial was added DMSO and sucrose solution (Fisher Chemical, CSA grade) to a final concentration of 10% (v / v) and 200 mM (v / V) respectively (Bui *et al*., 2013); while in the other vial was added glycerol to a final concentration of 10% (v / v) (Hubálek, 2003). The cryopreservants were added slowly to the cryovials containing the cultures (Bui *et al*., 2013).

The cryovials were inserted into a polyethylene container with a screw cap filled with 125 ml isopropanol (Bui *et al*., 2013). Isopropanol enable a freezing rate of approximately −1 °C min^{-1} (Shiraishi, 2016). This container was kept in a freezer at -80 °C for 120 hours, which is the

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approximate time for replenishment of stocks during semi-continuous cultures with harvest every five days. After this time, the container was removed from the freezer and the cryovials were floated in a beaker filled with water (~ 500 ml at 25 °C) (Bui *et al.*, 2013), 4 cryovials at a time. The thawed cell cultures were gently poured into sterile Falcon tubes containing 49 ml of Guillard's f/2 medium at room temperature and left unmixed for 1 h and subsequently carefully inverted 5 times (Bui *et al.*, 2013). Falcon tubes were kept in the dark for 24 hours at 30 °C. After this time, the microalgae were cultivated at 30°C and photoperiod 12:12 for 6 days. At the end of the culture period the algal batches were filtered using Whatman GF/F and GF/D glass fiber filters (~ 0.7 µm and 2.7 µm pore size respectively) according to the size of the microalgae. The growth rate was determined and expressed as mentioned above.

Total Lipid

Total lipid content was determined following the protocol described by Christie (1994), by extraction of the filtered microalgae biomass from the cryopreservation test using a mix of chloroform, dichloromethane and methanol (1:1:1). The ethereal extract was determined by weight difference.

Statistical analysis

Statistical analysis was performed by two-way analysis of variance (ANOVA) using Statistica v7.0.61.0 Software. Normality of distribution was check with a Shapiro test. A p value of <0.05 was consider as statistically significant, and Post-hoc analysis was done with a Tukey test to determine significance. The total lipid was analyzed by nonparametric Kruskal.Wallis and Median test as the data showed non-normal distribution.

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Result and discussion

The genetic identification conducted by 18S rRNA partial sequences and the SILVA database matched the morphological identification with the most probable genera to be *Chorella* sp. (M2), *Chlorella* sp. (M6), *Scenedesmus* sp. (R3), and *Coelastrella* sp. (A2). Moreover, to determine an association between our sequences and other characterized algal sequences from the GenBank database, an UPGMA consensus tree was constructed (Fig. 1). Consensus sequence percentage showed a 90% of similarity between M2 and M6 in nucleotide alignment. Sequences from M2 and M6 had a 67.9% of similarity to members of the genera *Chlorella*. Overall, M2 and M6 clustered within members of the Chlorellaceae family, and included genera such as *Chlorella* and *Parachlorella*. These results would point out the relationship among members of this family as previously reported (Krienitz *et al*., 2004; Bock, Pröschold & Krienitz, 2011) On the other hand, R3 and A2 clustered with members of the Scenedesmaceae family, including *Coelastrella*, *Scenedesmus* and *Desmodesmus* (Fig. 1). There was a 64.1% of similarity in terms of nucleotides between R3 and A2. Both genera were not completely resolved using phylogenetic analysis (Fig. 1). The small discrepancies to define genera in the clades may be due to partial sequences obtained in this study. Nevertheless, bootstrap values were higher than 50% for these branches. We also support our findings with the morphological identification conducted for all algal isolates from this study, including R3 and A2. In other phylogenetic studies, members of *Coelastrella* and *Scenedesmus* fall within a combining node, which may indicate a common lineage between both genera (Baytut *et al*., 2013; Jiang *et al*., 2014).

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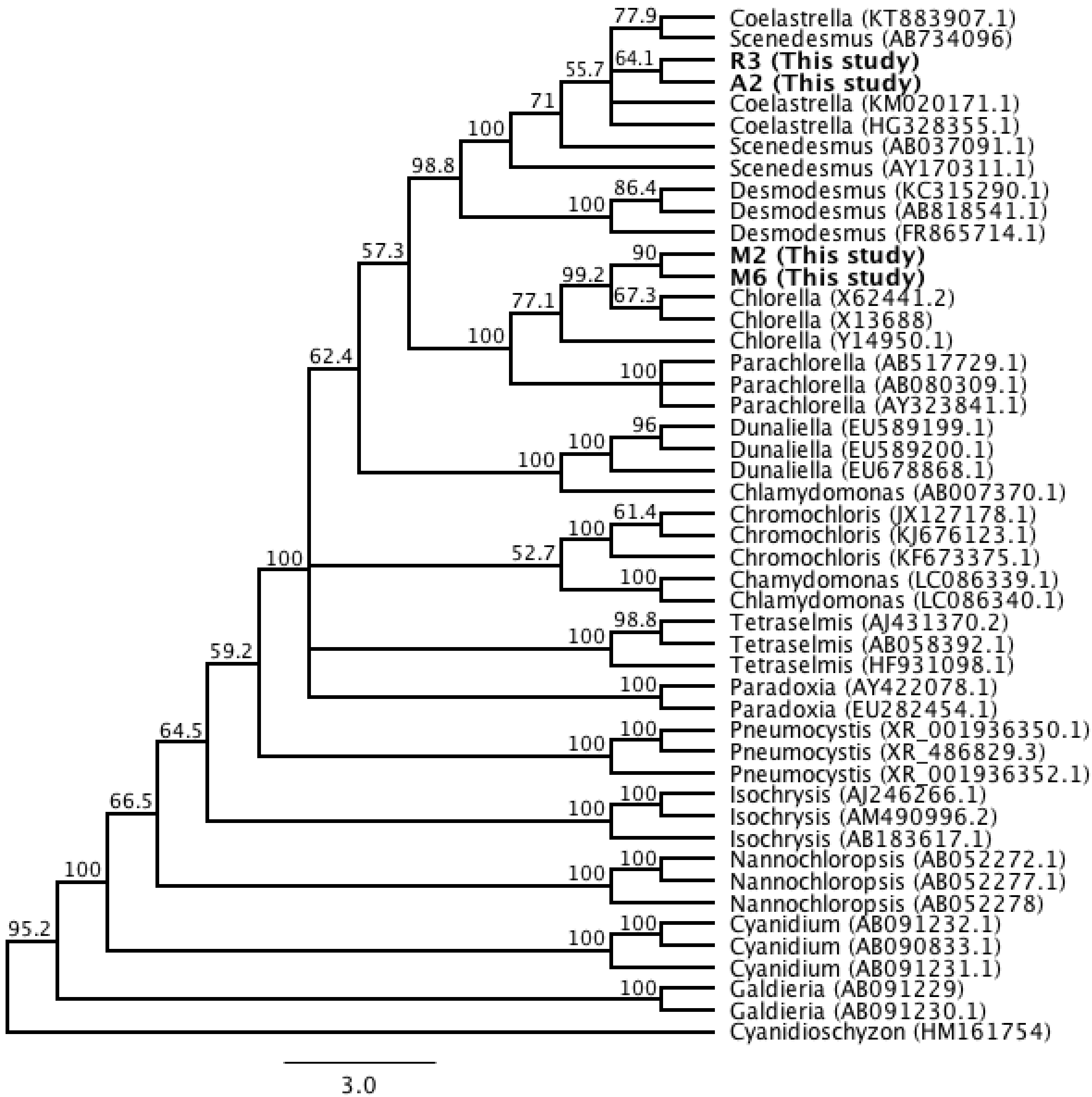

Figure 1.- Unweighted pair group method with arithmetic mean (UPGMA) consensus tree of the 18S rRNA sequences isolated in this study and other representative algal sequences retrieved from the National Center for Biotechnology Information (NCBI) database. Numbers in the branches represent bootstrap values from 1,000 replications. Numbers in parentheses are the GenBank accession identification numbers.

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Growth rate using Chloramphenicol

All microalgae strains cultured with chloramphenicol showed a lower growth rate than the negative controls (two-way ANOVA, F =26.68,p<0.001,df =2; post-hoc in Table 1) (Fig. 2). This demonstrate that CAP can inhibit microalgae growth, especially *Scenesdesmus* sp. R3, which showed a 65% decrease in its growth rate in comparison to its control culture (Fig. 2). Similar results have been reported by Lai *et al.* (2009), where concentrations 20 - 40 mg L^{-1} of chloramphenicol inhibited growth of *Chlorella pyrenoidosa*, which is near the range used in the present study (50 mg L^{-1}). However, CAP in lower concentrations (0.5 a 12.0 mg L^{-1}) have been shown to have no significant effect in the growth of *C. pyrenoidosa* and *Isochrysis galbana* (Campa-Córdova *et al*., 2006; Lai *et al*., 2009). This effect has been attributed to the CAP acting as an inhibitor of photosynthetic oxygen evolution and as well as an inhibitor of protein synthesis in chloroplasts, affecting the chlorophyll synthesis in photosynthetic organisms. Seoane *et al.* (2014) reported one decreased in size and in chlorophyll a content, but detected an increase in chlorophyll a fluorescence in microalga *Tetraselmis suecica*, this could be due to an inhibitory effect localized on the oxidant side of mitochondria. This effect is short-term, several studies showed that microalgae ad resistance to chloramphenicol when Additional to the toxic effect of chloramphenicol on photosynthesis, the high antibiotic concentration used in the present study could eliminate the bacteria associated to the freshly isolated strains, which could contribute of the observed negative effect when the chloramphenicol concentration increases. It is known that microalgae live in synergism with certain bacteria, interacting between each other, through nutrients interchange, signal transduction, genes transference and others (Kouzuma & Watanabe, 2015). In a study by Lubarsky and collaborators (Lubarsky *et al*., 2010), it is shown that the microalgal biomass was significantly lower in the axenic cultivate (antibiotics added) as

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compared to the cultivate associated with bacteria. However, a more detailed study would be necessary to access the actual cause of the observed effect.

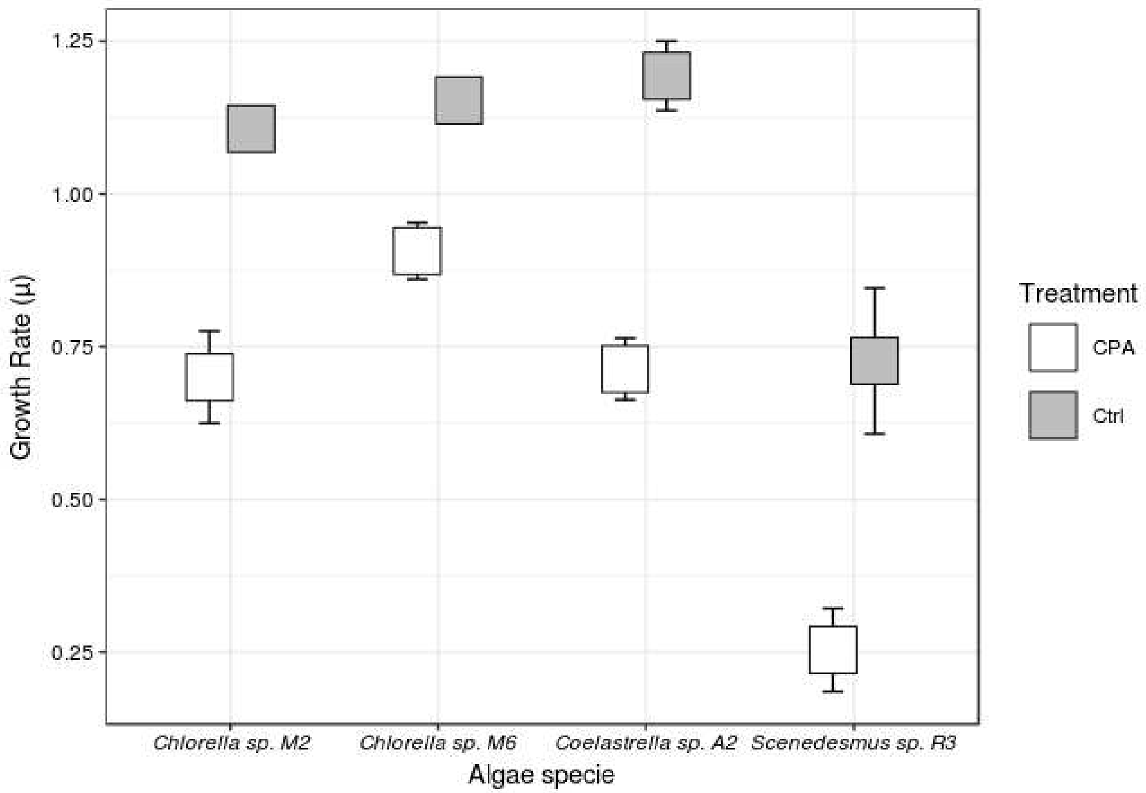

Figure 2.- Growth rate of microalgae in culture with chloramphenicol (CPA) in final concentration of 50 mg L^{-1} and Negative control culture without antibiotic per strain (Ctrl).

Cryopreservation Test

The *Colaestrella* sp. A2 strain did not show any growth after cryopreservation with both DMSO-sucrose or glycerol, which may be due to its very low initial stock before the process, which was below the recommended for this method (Bui et al., 2013) ($<2 \times 10^7$ cell ml^{-1}). *Scenedesmus* sp. R3 showed no significant difference in growth rate between before and after of cryopreservation, unlike other 2 strains. Saadaoui *et al*. (2016) reported that *Scenedesmus* cells are rapidly recovered after 1 month storage in liquid nitrogen using dimethyl sulfate as cryoprotectant.

Chorella sp. M2, *Chlorella* sp. M6 and *Scenedesmus* sp. R3 showed no significant difference in growth rate between the two cryopreservants (two-way ANOVA, F =9.63,p 0.0018,df =2; post-hoc in Table 1) (Fig. 3, Top). This may be due to that in certain microalgae strain the combination of permeable and impermeable cryopreservants such as DMSO-sucrose, is necessary for preservation during freezing, because sucrose increases solution osmolarity causing cell dehydration, working synergistically with DMSO to minimize intracellular ice damage. While glycerol increases the total concentration of solute, consequently reduces the amount of ice formed(Bui *et al*., 2013). Moreover, Glycerol decrease the freezing-point of water and biological fluids by colligative action (glycerol/water to a minimum of -46°C), also, it prevent eutectic crystallization (Hubálek, 2003).

Total Lipid

The total lipid were analyzed in the biomass of microalgae strain with similar growths in both cryopreservants. Higher lipid content was observed in biomass cryopreserved with glycerol, thus, in *Chorella* sp. M2 y *Chorella* sp. M6, it is twice as high than in biomass cryopreserved with DMSO + sucrose (Chi-Square= 6,300000 df = 2 p = 0,0429) (Fig. 3, Bottom). This might be due to the fact that some algae can utilize glycerol as a carbon source, as the *Chlorella vulgaris* achieved maximum lipid productivity in glycerol supplemented culture medium (Sharma *et al*., 2016), this could be the reason of the increase in lipid content observed.

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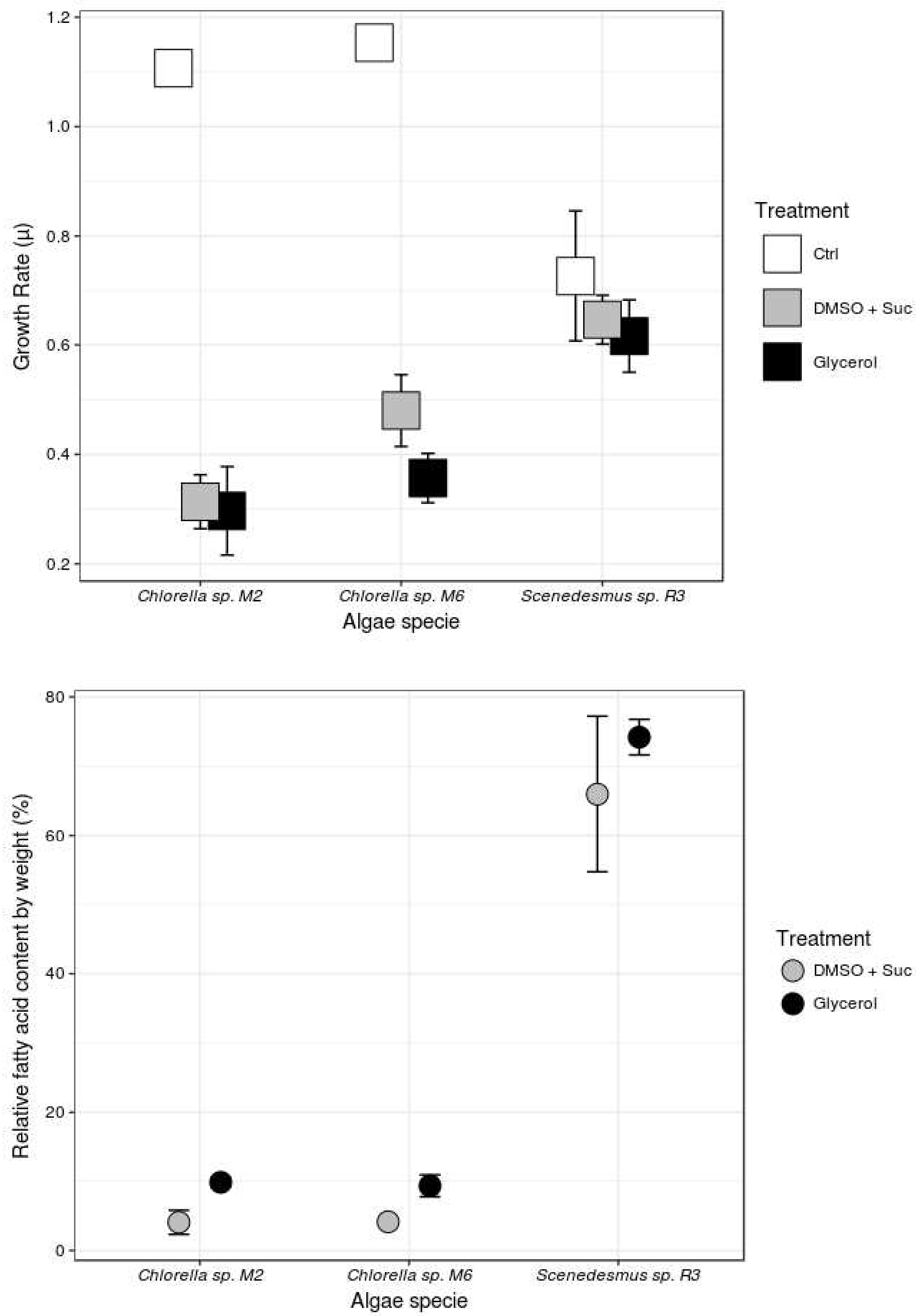

Figure 3.- Top: Microalgae growth rate after cryopreservation with dimethyl sulfoxide and sucrose (DMSO + Suc) and Glycerol, plus a control with no cryopreservation (Ctrl). Bottom: Total lipids of microalgae after cryopreservation with with dimethyl sulfoxide (DMSO + Suc) and Glycerol.

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Table 1.- Result of the post-hoc statistical analysis of the antibiotic chloramphenicol (CPA) and its negative control (NC); Cryopreservation treatments with Dimethyl sulfoxide + sucrose (DMSO+S) and Glycerol (Gly). All microalgae combinations are listed. The values given are p values. Values with p<0.05 are presented in bold.

Microalgae	CPA	NC	DMSO+S	Gly
Chlorella sp. M2 – *Chlorella* sp. M6	**0,0124**	0,8426	**0,0199**	0,5385
Chlorella sp. M2. – *Scenesdesmus* sp. R3	**0,0003**	**0,0008**	**0,0007**	**0,0024**
Chlorella sp. M2 – *Coelastrella* sp. A2.	0,9950	0,4770		
Chlorella sp. *M6* - *Scenesdesmus* sp. R3	**0,0002**	**0,0004**	**0,0186**	**0,0063**
Chlorella sp. *M6* - *Coelastrella* sp. A2.	**0,0153**	0,9004		
Scenesdesmus sp. *R3*- *Coelastrella* sp. *A2*	**0,0003**	**0,0003**		

Conclusion

The cultivation process can affect the microalgal productivity of tropical freshwater microalgae isolates, as chloramphenicol can reduce microalgae growth when used at concentration of 50 mg L^{-1}; however, the effectivity of using lower concentrations of CAP is the same in the elimination of bacteria from the cultures is still to be demonstrate. It is still necessary to be determined if the observed deleterious effect of high CAP concentration in algal growth rate is long lasting in the strains after they are subsequently cultured.

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Both DMSO-sucrose and glycerol are effective microalgae cryopreservants in most cases, especially in *Scenedesmus*. However, the above results show that cryopreservation might not be suitable for all algae, like *Coelastrella* sp. A2.

The lipid content of the algae is significantly affected by the cryopreservation method, so species like *Chlorella* sp. M2 and *Chlorella* sp. M6 should be cryopreserved with glycerol when cultivation to obtain a higher lipid gain per cell. As with the antibiotics, is necessary to further determine if the negative effects of DMSO-sucrose in the algal lipid content are whether temporary or carried forward in future cultures.

The results highlight the relevance of choosing an appropriate method for obtaining axenic cultures and their posterior storage as the methods use can severely affect the biological performance of different species of tropical freshwater microalgae isolates, although is still to determine if this effects are long lasting.

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