Growth of Diatom Fistulifera Alcalina in Bacterial Co-culture and Comparative Mitogenomics of Fistulifera Species

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GROWTH OF DIATOM *FISTULIFERA ALCALINA* IN BACTERIAL CO-CULTURE
AND COMPARATIVE MITOGENOMICS OF *FISTULIFERA* SPECIES

A dissertation submitted in partial fulfillment of
the requirements for the degree of
DOCTOR OF PHILOSOPHY
in
BIOLOGY
by
Erwin David Berthold

2021
To: Dean Michael R. Heithaus  
College of Arts, Sciences and Education

This dissertation, written by Erwin David Berthold, and entitled Growth of Diatom *Fistulifera alcalina* in Bacterial Co-Culture and Comparative Mitogenomics of *Fistulifera* Species, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this dissertation and recommend that it be approved.

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Kathleen Rein

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Date of Defense: March 17, 2021

The dissertation of Erwin David Berthold is approved.

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College of Arts, Science and Education

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Andrés G. Gil  
Vice President for Research and Economic Development  
and Dean of the University Graduate School

Florida International University, 2021
CHAPTER 1


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CHAPTER 2


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DEDICATION

I dedicate this work to the advisors that have especially shaped the scientist I am today, Dr. Haywood Dail Laughinghouse IV, Dr. Evelyn Eileen Gaiser, and Dr. Miroslav Gantar. I would also like to dedicate this work to my loving sisters who have supported me throughout this journey, Cyndie “Big Mama” and Tadjie “TaDooDoo” Berthold and my parents that gifted me my first microscope and supported all my decisions, Michele “Mimiche” Roux Berthold and Erwin “Pousson” Berthold. And finally, I would like to dedicate this work to my country of birth, Haïti, because there just aren’t enough black scientists. Finally, I want to dedicate my work to Mother Nature, and pursuing ways to ameliorate the ways in which humans interact with her.
ACKNOWLEDGMENTS

I would like to thank Dr. Miroslav Gantar for giving me the fundamental skills that have made most of my work possible, especially my culturing skills. I thank Dr. Gantar for helping me become the scientist I am today by providing me both the responsibility and freedom in the lab to learn about algae. Without Dr. Gantar, I might never have gotten interested in algal biofuels and medicinal compounds. I would like to thank my advisor Dr. Haywood Dail Laughinghouse IV for his financial, emotional, educational and professional support throughout my dissertation. Dr. Laughinghouse opened doors that would have otherwise been off limits and personally educated me on a breadth of subjects related to algae including and not limited to algal treatment, identification, culturing, and fundamental phycological skills. Without Dr. Laughinghouse, my focus and personal interest in respecting algal species on an individual basis would be naught. I would like to thank my advisor Dr. Evelyn E. Gaiser for her support and sticking by my side through hardships. Without the help of Dr. Gaiser, I would not be where I am today both educationally and professionally. I want to thank Dr. Gaiser for keeping me and my work grounded and always headed in the right direction: Although our time was short, I expect there is much to be said for the future. I would also like to thank Drs. Krishnaswamy Jayachandran and Kateel Shetty for supporting me and producing and publishing Chapter 1. I would like to also thank Dr. Braham Dhillon from University of Florida for his support and dedication to helping me write and submit Chapter 4. Finally, I want to thank my committee members Dr. Kathleen Rein and Dr. Mauricio Rodriguez-Lannetty for believing in me and for their support in my progress.
ABSTRACT OF THE DISSERTATION

GROWTH OF DIATOM FISTULIFERA ALCALINA IN BACTERIAL CO-CULTURE
AND COMPARATIVE MITOGENOMICS OF FISTULIFERA SPECIES

by

Erwin David Berthold

Florida International University, 2021

Miami, Florida

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Diatoms are excellent biological models of growth and intracellular oil
generation. The productivity and compounds of diatoms, especially oils, support aquatic
food chains and human medical and industrial needs. The qualities that made diatoms
prolific producers, specifically diatom physiological features such as growth rates with
intracellular lipid storage in alkaline environments, are however poorly understood.
Another physiological aspect that remains unexplored is the effects of bacteria on the
growth and lipid production of alkaliphilic diatoms. More studies, especially co-cultures,
are needed for advances in diatom biology and strain performance for the algal
biotechnological field. Besides physiology, diatom genetics using next-generation
methods are used to reveal the basis for diatom success in growth and productivity,
though more genomic data and comparative studies are needed to fully uncover these
mechanisms in diatoms. To improve our understanding of alkaliphilic diatom physiology
and genetics, specifically we need to understand 1) what stimulates diatom successful
growth in alkaline environments and in culture, 2) diatom biodiversity especially at the
species level, 3) algal physiology in the context of how diatom productivity may be enhanced by bacteria, and 4) the biological basis for the extraordinary growth rates of diatoms and their success in alkaline environments.

To explore ways in which we can improve our understanding of alkaliphilic diatom physiology and genetics this dissertation aimed to first isolate alkaliphilic algae and explore their alkalinity optimums along with their growth rates and lipid production. A high lipid producing diatom *Fistulifera alcalina* was isolated and characterized using 18S rRNA and rbcL molecular phylogeny. To further explore the diversity within *Fistulifera* and simultaneously exploit diatom microbiomes, Lake Okeechobee was sourced for alkali-tolerant to alkaliphilic bacteria associated with diatoms to co-culture with *F. alcalina*. Results indicated that alkaliphilic bacteria like *Bacillus horikosshi* were effective in modulating growth and lipid content of *F. alcalina*. To explore the basis for the extraordinary growth rates in *F. alcalina* and its’ success in alkaline environments, the mitochondrial genome was sequenced and compared to *F. solaris*. Results indicate that alkaliphilic diatoms may have streamlined genomes and are impetus for further genome sequencing and physiological studies.
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<th>Description</th>
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<tbody>
<tr>
<td>18S rRNA</td>
<td>small subunit of ribosomal 18S RNA gene sequence</td>
</tr>
<tr>
<td>BI</td>
<td>Bayesian Inference</td>
</tr>
<tr>
<td>CDS</td>
<td>coding sequence</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming units</td>
</tr>
<tr>
<td>DIH₂O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DIC</td>
<td>differential interference contrast [microscopy]</td>
</tr>
<tr>
<td>DIC</td>
<td>dissolved inorganic carbon</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DEFT</td>
<td>direct epifluorescent microscopy</td>
</tr>
<tr>
<td>DOC</td>
<td>dissolved organic carbon</td>
</tr>
<tr>
<td>DW</td>
<td>dry weight</td>
</tr>
<tr>
<td>EFM</td>
<td>epifluorescent microscopy</td>
</tr>
<tr>
<td>EPS</td>
<td>extracellular polymeric substances</td>
</tr>
<tr>
<td>EAA</td>
<td>Everglades Agricultural Area</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FAMEs</td>
<td>fatty acid methyl esters</td>
</tr>
<tr>
<td>FAs</td>
<td>fatty acids</td>
</tr>
<tr>
<td>GC-MS</td>
<td>gas chromatography-mass spectrometry</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoproteins</td>
</tr>
<tr>
<td>HGTs</td>
<td>horizontally gene transfers</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>LDs</td>
<td>lipid droplets</td>
</tr>
<tr>
<td>LM</td>
<td>light microscopy</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>MCs</td>
<td>microbial communities</td>
</tr>
<tr>
<td>MeCoCos</td>
<td>metabolically cohesive consortia</td>
</tr>
<tr>
<td>mL</td>
<td>milliliter</td>
</tr>
<tr>
<td>ML</td>
<td>Maximum Likelihood</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>MZ10</td>
<td>modified Zarrouk’s medium at pH 10</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PA</td>
<td>palmitoleic acid</td>
</tr>
<tr>
<td>PUFFAs</td>
<td>polyunsaturated fatty acids</td>
</tr>
<tr>
<td>rbcL</td>
<td>ribulose bisphosphate carboxylase large chain</td>
</tr>
<tr>
<td>rpm</td>
<td>rotation per minute</td>
</tr>
<tr>
<td>RubisCO</td>
<td>ribulose-1,5-bisphosphate carboxylase/oxygenase</td>
</tr>
<tr>
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<td>scanning electron microscope</td>
</tr>
<tr>
<td>TAGs</td>
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GENERAL INTRODUCTION

Diatoms are proficient at sequestering carbon dioxide, generating lipids, and have high doubling rates, making them appealing organism models for biological research as well as biotechnological applications. Cultivation of algae, especially diatoms, is a proven source of renewable energy and valued fat and protein products (Jacob et al 2020; Oliver et al. 2020). However, the combined qualities that make diatoms ideal contenders for large scale cultivation are not completely understood. To improve our understanding of the drivers of diatom productivity we need to understand: 1) what stimulates diatom successful growth in the environment and in culture, 2) diatom biodiversity especially at the species level, 3) algal physiology in the context of how diatom productivity may be enhanced by bacteria, and 4) the biological basis for the extraordinary growth rates of diatoms and their success in alkaline environments. To address these needs, the goals of the dissertation are as follows:

To explore the basis for diatom growth success in the environment and in culture, extremophilic algae like alkaliphiles are good models since they are capable of growth where other cannot. Few strains of alkaliphilic algae, however, are available and applied towards research. To satisfy the necessity for alkaliphilic algae the primary goal of this dissertation was to 1) bioprospect Lake Okeechobee for alkali-tolerant/alkaliphilic strains of algae (especially diatoms) capable of robust growth in extreme alkaline environments.

Characterizing diatoms on a species level is another crucial aspect in describing diatoms since no two species are alike, and different algal species can widely diverge in physiology. To explore diatom biodiversity at the species level, the next goal was to 2) characterize a diatom strain through molecular phylogenetic and morphological means.
In combination with bioprospecting novel alkaliphilic diatom species and exploring their unique characteristics, investigating the mechanisms of growth is another aspect of understanding diatom physiology. To investigate the mechanisms of diatom growth the third goal was to 3) evaluate the effects of heterotrophic bacteria on the growth rate, lipid content and productivity of a diatom. In addition to these physiological inferences, applying genetic methods are also invaluable to investigate the biological basis for diatom diversity, growth rates, and success in alkaline environments. The final goal of the dissertation is to 4) explore the mitogenome of diatoms, and through comparative genomics elucidate potential genetic sources that render certain species successful biological producers. Sequencing new genomes and using preexisting genomes in comparative analyses are powerful methods of pushing our understanding of diatom genetic architecture, molecular phylogenetic placement, and ultimately organelle evolution.

The research unites classical methods of microbiological selection and isolation, contemporary microscopic analyses of diatom morphology and taxonomy, molecular phylogenetic methods and modern next generation sequencing in describing a novel diatom species, *Fistulifera alcalina*, and culturing techniques to advance prospects for mass cultivation.

*What are algae and why study diatoms?*

Algae are a polyphyletic group of macro- to microorganisms that mostly undergo chlorooxygenic photosynthesis to form simple sugars through photon capture using pigments, with oxygen as byproduct. Algae are ubiquitous organisms in aquatic [and
Diatoms are a lineage within the stramenopiles that are adaptable organisms since they inhabit marine, fresh, and terrestrial environments from tropical to polar regions, and represent an especially taxonomically diverse group of microorganisms (Malviya et al. 2016; Giordano et al. 2005).

Aside from their cosmopolitan tendency, diatoms hold great ecological significance in their respective habitats. Diatoms are the foremost primary producers in oceans and support aquatic food webs (Malviya et al. 2016) by providing proteins, lipids, carbohydrates, vitamins, and micronutrients (Long et al. 2019). Diatoms are prolific generators of fats and proteins and often applied to supplement food for animal husbandry and aquaculture (Sprague et al. 2016). Diatoms also have comparatively higher growth rates than other algae which make them ideal candidates for industrial cultivation (Hildebrand et al. 2012). Diatoms are largely involved in the global cycling of oxygen and silica (Nelson et al. 1995) and are especially effective at assimilating CO₂ (Beardall and Raven 2020). The basis for diatom carbon sequestering quality arises from the CO₂ concentrating mechanisms (CCM) or the collection of intracellular dissolved inorganic carbon (DIC) used for photosynthesis. Through the CCM, diatoms are able to concentrate DIC at concentrations much higher than the surrounding environment, which creates a flux of carbon through the four chloroplast membranes and to RubisCO (ribulose-1,5-bisphosphate carboxylase/oxygenase) for fixation (Tsuji et al. 2017). The biological efficiency of diatoms makes them ideal model organisms to study carbon sequestration and the biosynthesis of compounds (Trobajo et al. 2014; Sethi et al. 2020).
Diatoms can be oleaginous (oil-producing), especially dense in triacylglycerides (TAGs) (Figure 1). The intracellular TAGs are assembled into lipid droplets (LDs) and largely used for storage of energy and other compounds including pigments (Pick et al. 2019). In a large evolutionary context, diatom LDs are considered an ancient and conserved organelle found in most eukaryotes that evolved from LECA (Last Eukaryotic Common Ancestor) (Leyland et al. 2020a).
Figure 1 Brightfield microscope images of diatoms isolated from diverse South Florida habitats demonstrating the ability of diatoms to accumulate large intracellular lipid droplet(s) within individual cells (black arrows).
Lipid droplets also represent a significant aspect of diatom physiology. To date, general scientific consensus suggests LDs are not just a storage receptacle, but an organelle and central metabolic complex that is in tune with environmental and cellular cues and involved in coordinated crosstalk between multiple cellular compartments (Leyland et al. 2020b). Proteomics of diatom LDs from *Phaeodactylum tricornutum* Bohlin and *Fistulifera solaris* S. Mayama, M. Matsumoto, K. Nemoto & T. Tanaka have revealed a multitude of proteins, some shared between different species, that maintain LD related function and are localized to the LDs (Nonoyama et al. 2019). The coordinated shuttling of proteins and fatty acids to and from the LDs help buffer diatom energetic needs within a fluctuating environment. Nutrient deficiencies such as nitrogen and phosphorus depletion and other environmental fluxes including temperature and light affect LD induction or degradation (Levitan et al. 2015; Remmers et al. 2018; Almaeyda et al. 2020). In addition to shaping lipid biosynthesis and ultimately hydrolysis, nutrient deficiencies, diurnal cycles, and physical perturbations affect different metabolic processes which in turn alter the proportions, classes and/or lipid species produced (Řezanka et al. 2020). Understanding the mechanics of environmental variables and the consequent shaping of lipid biosynthesis is a crucial aspect of diatom research. Most importantly, additional studies of new model organisms are also needed to expand how we understand the functions of lipid biosynthesis (Leyland et al. 2020a).

Biosynthesis of TAGs in diatoms, like plants and other algae, involve both the plastid and the endoplasmic reticulum (ER) (Cagliari et al. 2011). Diatom lipid biosynthesis takes place within the single cells of diatoms, while plants concentrate TAGs within seeds. Biosynthesis of TAGs occurs within the chloroplast with acetyl-CoA
(acetyl coenzyme A) as starting material. Acetyl-CoA is used to support the metabolic pathways for lipids, proteins, and carbohydrates and more recently pathways for sterols (Maréchal and Lupette 2020). Understanding how the flow of carbon energy in the form of Acetyl-CoA is dictated across the various metabolic pathways is crucial to grasp diatom physiology.

Unlike plants, diatoms and other stramenopile algae are capable of synthesizing large quantities of PUFAs (polyunsaturated fatty acids) with very long carbon chains (Lim et al. 2020). Fatty acids with carbon chains higher than C:18 are produced in several plant (Simopoulos 2002) species like sea buckhorn berries, walnuts, macadamia seeds, and hemp seeds, but do not reach the quantities observed in some marine diatom species (Yang and Kallio 2001; Racine and Deckelbaum 2007). The very long carbon chain produced by diatoms, especially marine, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential not only in the diets on fish but also a vital supplement for humans (Popovich et al. 2020; Marella and Tiwari 2020).

Diatoms that generate large lipid quantities are pursued for valued compounds able to sustain human oil applications in nutraceutical, pharmaceutical, and industrial trades (Ward and Singh 2005; Yang and Xie 2020). Compared to other algal species such as green algae and cyanobacteria, diatom TAGs are mainly composed of PUFAs (Lang et al. 2011; Yi et al. 2017) and may make up to 30-60% or more of the diatom cell weight (Tanaka et al. 2015; Berthold et al. 2020a). Fatty acids (FAs) such as EPA or omega-3 (ω-3), and ω-7 are an attractive commodity not only for their medical applications (Morse 2015) but also for their appeal as marketed vegan alternatives to fish oil. Sourcing oils from fish competes with global fish supply for human consumption and raises the
demand for farm raised fish, which already imposes health and environmental burdens (Mavraganis et al. 2020). Contamination of fish oils with heavy metals is another common disadvantage (Javed and Usmani 2019). Extracting oils from fish may also seem unnecessary since fish derive most EPA and DHA from their algal diet (Ofosu et al. 2017). Diatom oils are an alternative to supplement current and emerging uses of ω-7, palmitic, EPA, and DHA and to help reduce the demand and burden on aquacultural and agricultural systems (Knothe 2010). There is great ecological and biotechnological importance to diatom compounds, and more research geared towards progressing and improving our understanding of diatom biology especially in the context of lipid biosynthesis is needed (Sunaga et al. 2015; Khan and Fu 2020).

**Diatom ecological and physiological significance**

Diatoms are a powerhouse of lipid generation and the intracellular LDs produced are not stagnant storage particles but complex and dynamic hubs of communication (Leyland et al. 2020b). Diatom LD composition and structure are continuously altered in response to a constant flow of environmental and cellular cues. In nutrient replete environments, diatoms proliferate and generally do not focus energy into forming lipids. In response to limitations of nutrients or physiological requirements such as light or optimal temperature, lipids are generated. Nutrient deprivation or light and temperature changes are well-studied aspects of diatom physiology in the context of lipid biosynthesis, but the effects of bacterial presence on lipid biosynthesis requires further exploration (Liu et al. 2020). Observing the effects of bacteria on diatom growth and lipid
biosynthesis can explain unknown mechanisms of diatom lipid biosynthesis previously not observed in nutrient deprivation or other physiological treatments.

Relationships between phytoplankton, especially diatoms, and bacteria are prevalent in marine and freshwater communities and extensively contribute to the functioning of these environments (Field et al. 1998; Sarmento and Gasol 2012). These complex microbial communities (MCs) influence large-scale global cycles of nitrogen, phosphorus, and other essential nutrients (Jørgensen 2006). Algae and bacteria have long occurred in aquatic habitats and closely interact (Armbrust et al. 2004; Bowler et al. 2008; Schönknecht et al. 2013). In combination with insight into the biogeochemical cycling of aquatic environments, research is now revealing the intrinsic molecular workings of algal and bacterial relationships (Liu et al. 2020). Though algal and bacterial coupling is thought to be straightforward where a photo-oxygenic organism benefits from one that respires, the relationship is often entangled in a complex combination of reciprocal compounds. Previous observations of single metabolites and their effects on algal growth has paved the way for a more wholesome concept of the generation, storage, and reciprocal release of numerous metabolites that influence algal-bacterial communities’ growth and predominance (Cirri and Pohnert 2019).

Algal and bacterial classical interactions include mutualism, commensalism, and parasitism (Ramanan et al. 2016). Within these interactions are many mechanisms in which bacterial and algal cells communicate, such as the exchange of extracellular compounds and quorum sensing. These communications can happen through cellular attachment or involve the extracellular cycling of specific compounds and elements including nutrients, dissolved organic carbon, vitamins, phytohormones, and secondary
compounds (Lau et al. 2007; Tang et al. 2010; Teplitski and Rajamani 2011; Bagwell et al. 2016; Yang et al. 2016). These compounds can be species-specific and dependent on the peripheral mucilage region of algae cells, or phycosphere (Bell and Mitchell 1972; Sapp et al. 2007) where extracellular polymeric substances (EPS) are often found. The communication between algae and bacteria may even be obligatory where diatom aggregation and biofilm formation, for example, are dependent on bacterial cell contact and exudates (Schnurr and Allen 2015; Windler et al. 2015; Yang et al. 2016).

To help explain the phenomena of algal and bacterial relationships, the recent concept of a self-constructed MC that optimizes its functions in relation to metabolism is a more comprehensive take on microbial interactions. A Metabolically Cohesive Consortium (MeCoCo) is a MC that functions together by both producing and using resources in a sustained and reproducible manner (Pascual-García et al. 2020). The MeCoCo members partake in specialized resources and roles which avoid competitive interactions and limit generalists. In the context of limiting generalists, microbial and algal relationships are not reduced to single interactions like mutualism and commensalism, but rather extended to include all relationships as a function of the community, individual community members, and the specialized resources available.

The MeCoCos concept has ecological, evolutionary, and biotechnological significance. In an ecological context, MeCoCos are established communities that can be applied to explain ecosystem dynamics, such as interactions between opposing MeCoCos. And though MeCoCos are generally centered around exchange of metabolites, the genes present within a community largely play a role in the refinement and
stabilization of MeCoCos (Hansen et al. 2007), and the evolutionary dependence between participating members.

The relationship between diatoms and bacteria is also an important consideration in biotechnological tool development (Marella et al. 2020). Since bacterial presence is key to the growth and success of algae, microbial interactions with oleaginous algal species may be particularly revealing of the physiological basis for diatom-bacterial interactions (Gonzales and Bashan 2000; Park et al. 2008; Amin et al. 2015). Bacteria have shown promising effects on algal growth rates and lipid production (Ward and Singh 2005; Natrah et al. 2014; Berthold et al. 2019; Liu et al. 2020; Kim et al. 2020; Marella et al. 2020). Accelerating diatom growth rate is an ultimate goal to scale up production (Hannon et al. 2010) that is facilitated through an improved understanding of diatom physiology, phylogeny, and genetics (Sunaga et al. 2015; Tanaka et al. 2015).

Elucidating the fundamental relationships between algae and bacteria often require physiological and modern molecular genomic scrutiny (Cooper et al. 2015). The abundance, inter-dependence, reciprocal functions, and evolutionary relationships between diatoms and bacteria in the environment are indispensable aspects of algal biology and often requires next generation sequencing and modern molecular methods for a deeper understanding at the genetic level (Amin et al. 2012; Ramanan et al. 2016).

**Diatom genetics and evolution**

Diatoms are unique not only for their silica frustule but also their genetic makeup compared to other photosynthetic organisms including other eukaryotic organisms and cyanobacteria. As an evolutionary product of a secondary endosymbiosis event, the
Diatom cell is an assembly of a photosynthetic red eukaryote assimilated into a nonphotosynthetic eukaryote (Keeling 2013). Diatom uniqueness is an evolving complexity since their evolution is now extended to include green algae-assimilated genes through endosymbiosis events. Green algae genetic remnants are commonly found within diatom genomes (Deschamps and Moreira 2012; Dorrell et al. 2017) and is altering the way scientists view diatom evolution (Morozov and Galachyants 2019).

Contemporary next-generation sequencing is a central aspect of modern algal molecular genetic studies. Whole genome analyses, including that of the mitochondria, plastid, and nucleus, have helped explain the ability of diatoms to inhabit even the most extreme environments from ice to wastewaters (Falciatore et al. 2020; Marella et al. 2020). Genomic data are especially helpful reference points when interpreting metagenomic data including proteomics and transcriptomics (Tanaka et al. 2015; Landa et al. 2017). Reference genomes are also the foundation for the de novo assembly of sequenced diatom genomes. Diatom genomes are powerful tools to explore genetic disparities among algal strains and uncover potentially novel genes useful in biotechnological advances of candidate strains. A greater understanding of algal genome organization and regulation is also necessary for targeted gene manipulation attempts to increase diatom productivity (Hildebrand et al. 2012). Whole-genome assembly and comparisons can be a prequel to a deeper understanding of microbial relationships in the realm of pushing limits of lipid productivity (Kim et al. 2020).
**Motivation for diatom research**

From our current understanding of algal physiology, increasing diatom growth rates, for example, entails simultaneously stimulating both biomass and lipid production. Counterintuitively, diatoms concentrate intracellular lipids when nutrients are depleted or in the late exponential phase of population growth, resulting in diminished biomass (Chisti 2007; Hannon et al. 2010). A reduction in biomass is generally caused by a redirection of energy to the production of the storage lipid compounds. To avoid diatom growth declines while maintaining high lipid yields, diatoms with high lipid productivities throughout their life cycle are good contenders for mass cultivation (Borowitzka and Moheimani 2013; Satoh et al. 2013). Some diatom species are already described as potential oleaginous candidates, including the marine diatom *Fistulifera solaris* (Matsumoto et al. 2014). Most algae cultivation candidates fall short of yet another challenge of mass cultivation - succumbing to contamination. Infestation of non-target species including grazers and fungi is one of the main obstacles in mass cultivation and frequently the critical step in production (McBride et al. 2014). Preventing contamination requires diatom strains with unique growth strategies such as extremophiles including halophilic, or alkaliphilic algae (Barnard et al. 2010) or using robust algae with a proclivity rather than an aversion for bacteria. Extremophilic algae, especially alkaliphilic algae, are an ideal cultivation strain since they grow where other organisms cannot, and naturally generate lipids (Gardner et al. 2011). A limited number of extremophile algal strains are accessible and used for marketable products as opposed to marine and freshwater algae (Mutanda et al. 2011). More strains with alkaliphilic and
extremophilic capacity are needed and can be attained through bioprospection of corresponding habitats.

To expand our knowledge on diatoms with unique growth potential, investigate the effects of beneficial bacteria on diatom lipid biosynthesis, and to explore the genetics of diatoms with these two qualities, the goals of this dissertation are to: 1) bioprospect Lake Okeechobee for alkali-tolerant/alkaliphilic strains of algae capable of robust growth in extreme alkaline environments, 2) characterize a diatom strain through molecular phylogenetic and morphological means, 3) evaluate the growth and lipid content and productivity of the diatom in co-culture with heterotrophic bacteria, and 4) explore the mitogenome of diatoms through comparative genomics to shed light on potential genetic sources that render certain species successful biological producers.

To isolate an alkaliphilic alga with high growth rate, Lake Okeechobee was chosen for bioprospection since these waters can reach alkaline levels. Formally describing organisms on an individual basis is a crucial aspect in biology since not all species, genera, and organisms are exactly alike, and ensures experimental duplication.

After describing the new species, the research aims to use natural occurring bacteria found in the phycosphere of diatoms isolated from the source lake of *F. alcalina* to determine its effect on the biomass and lipid productivity of *F. alcalina*. The bacteria found naturally occurring in the diatom cultures, and the isolated diatoms, can also provide valuable resources to study and understand the mechanism underlying microbial and algal relationships (Murray et al. 2020). To investigate the potential effects of bacterial presence on the physiology of *F. alcalina* the coupling of algae and bacteria is an effective strategy (Berthold et al. 2019; Liu et al. 2020).
Besides the physiology of diatoms, mitochondrial genomes of diatoms are powerful tools to investigate the genetic basis for the observed success of diatoms is comparative genomics. Diatom mitogenomes fill the missing gaps in our phylogenetic interpretations and strengthen the interpretations drawn on organelle evolution. The complete mitochondrial genome of *F. alcalina* is presented herein and subsequently compared to that of its sister species *F. solaris*. 
I. CHAPTER 1
Omega-7 producing alkaliphilic diatom *Fistulifera* sp. (Bacillariophyceae) from
Lake Okeechobee, Florida
Omega-7 producing alkaliphilic diatom *Fistulifera* sp. (Bacillariophyceae) from Lake Okeechobee, Florida

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ABSTRACT

Incorporating renewable fuel into practice, especially from algae, is a promising approach in reducing fossil fuel dependency. Algae are an exceptional feedstock since they produce abundant biomass and oils in short timeframes. Algae also produce high-valued lipid products suitable for human nutrition and supplement. Achieving goals of producing algae fuels and high-valued lipids at competitive prices involves further improvement of technology, especially better control over cultivation. Manipulating microalgae cultivation conditions to prevent contamination is essential in addition to promoting optimal growth and lipid yields. Contamination of algal cultures is a major impediment to algae cultivation that can however be mitigated by choosing extremophile microalgae. This work describes the isolation of alkali-tolerant / alkaliphilic microalgae native to South Florida with ideal characteristics for cultivation. For that purpose, water samples from Lake Okeechobee were inoculated into Zarrouk’s medium (pH 9-12) and incubated for 35 days. Selection resulted in isolation of three strains that were screened for biomass and lipid accumulation. Two alkali-tolerant algae *Chloroidium* sp. 154-1 and *Chlorella* sp. 154-2 were poor lipid accumulators. One of the isolates, the diatom *Fistulifera* sp. 154-3, was identified as a lipid accumulating, alkaliphilic organism capable of producing 0.233 g L\(^{-1}\) d\(^{-1}\) dry biomass and a lipid content of 20-30% dry weight. Lipid analysis indicated the most abundant fatty acid within *Fistulifera* sp. was palmitoleic acid (52%), or omega-7, followed by palmitic acid (17%), and then eicosapentanoic acid (15%). 18S rRNA phylogenetic analysis formed a well-supported clade with *Fistulifera* species.

**Key Words:** algae; alkaline; biofuels; bioprospection; lipids; nutraceuticals; palmitoleic acid; pharmaceuticals
1. INTRODUCTION

The demand for oleaginous algae for the biofuel, pharmaceutical, and nutraceutical industries is growing. Mass production of algae is a superior alternative over traditional crop and residual feedstock for biodiesel and bioproducts since algae have high growth and oil-accumulation rates, can sequester carbon dioxide, and can thrive on non-arable land and wastewater (Borowitzka and Moheimani 2013). When algae do not produce the required biofuel lipid profile, high-valued lipid compounds have proven indispensable towards nutraceuticals, pharmaceuticals, and cosmeceuticals (Ward and Singh 2005). A wide range of algal-derived lipids are produced for use in health supplements, food and animal feed additives, and have an immeasurable potential for the treatment of numerous human conditions (Zhang et al. 2010, Costa et al. 2012, Gantar et al. 2012, Xia et al. 2013).

Algae are emerging as one of the most promising and sustainable sources of fatty acids, including omega-3 (ω-3) and omega-7 (ω-7), which are directly extracted and marketed as vegan and vegetarian alternatives. Omega-7 fatty acids, or palmitoleic acid (16:1n7; PA), may be beneficial to the nutraceutical industry for its favorable effects on human health. PA is a promising compound for managing metabolic syndrome, a collection of symptoms including central obesity and glucose and insulin resistance, which is currently treated using several drugs with the additional risk of side effects. Preclinical and epidemiological studies have shown that anti-inflammatory and lipid lowering effects of PA are linked to the prevention of metabolic syndrome, which is associated with the risk of developing cardiovascular disease and type 2 diabetes. It is reported that PA reduces...
high-sensitivity C-reactive protein, triglycerides and low-density lipoproteins, while increasing high-density lipoproteins (HDL) (Bernstein et al. 2014, Morse 2015). Two major plant sources of PA include seeds of sea buckthorn (*Hippophae rhamnoides* L.) with 32% PA content and seeds of macadamia (*Macadamia integrifolia* Maiden et Betch) with 20 to 30% PA content (Saleeb et al. 1973, Yang and Kallio 2001). These plants are valuable sources with limited availability and therefore may not be sustainable if the demand for PA increases beyond the limited supply from plant sources.

Although the traditional extraction of PA from plants may not be sustainable, the effort to obtain PA from viable sources extends towards microalgae (Lenihan-Geels et al. 2013). Algae-derived PA can be achieved in larger quantities in shorter time frames than in plants by optimizing algal culture conditions (Zhu et al. 2016). The extraction of oils directly from the microalgae is superior over conventional methods since the main components of fish oil (ω-3 and ω-7) come from the algae in their diets (Ofosu et al. 2017). Extracting PA from algae avoids the disadvantages of using fish including metal contamination and the disagreeable taste and odor often associated with fish oils (Adarme-Vega et al. 2012). Moreover, sourcing omega fatty acids from fish may compete with global food supply, especially in a changing climate where fisheries’ productivity is challenged in sustained provision of stock (Core Writing Team et al. 2014). Overall, using algae as an alternative can supplement the current and emerging uses of ω-7 and help reduce the demand and burden on aquacultural and agricultural systems (Knothe 2010).

Although algae are a promising source of essential omega fatty acids, producing algae-derived biofuels and high value compounds is challenging considering the level of scaling-up required for mass production of high-quality product (Hannon et al. 2010). To
achieve large-scale algae-based production of compounds, it is imperative to have control over optimal conditions for highest yield. Control over biological and physicochemical properties of algae culture systems is essential for identifying and overcoming barriers to the scaling-up process. The primary issue associated with the success of large-scale microalgal cultivation is contamination (McBride et al. 2014). Algal cultures can become contaminated with herbivores and algal pathogens (viruses, bacteria, and fungi) and the main strain can be outcompeted for resources by fungi, bacteria, microbes, and other microalgal strains (Shunyu et al. 2006). Biological contamination reduces productivity and often results in culture failures and consequent loss of product. Despite the form of cultivation, whether open raceways or photobioreactors, biological pollutants are introduced through water input and gas exchange (Wang et al. 2013). To avoid the time and cost expenditure associated with culture contaminations, methods of prevention and eradication without interrupting microalgae growth are desired (Scott et al. 2010).

A primary approach in facilitating high biomass and lipid productivity while preventing contamination involves regulating the physicochemical and biological parameters of cultivation. Physicochemical methods of purifying culture contamination involve altering culture temperature or chemical treatment with possible negative effects on the target culture (Moreno-Garrido and Cañavate 2001). Biological methods for preventing culture contamination include the application of polycultures of algae, allelopathy, or selecting and using extremophile strains capable of enduring harsh cultivation conditions. Polyculture approaches can be designed where microalgal communities, with the desired product, are able to resist contamination (Smith and Crews 2014). While polycultures are plausible, they are difficult to construct as random
assemblages may introduce complexity (Weis et al. 2008). Contamination of algal cultures can be prevented by using strains that excrete secondary metabolites with inhibitory allelopathic effects against the contaminating organisms (Chiang et al. 2004). However, obtaining a strain that is both highly productive in terms of lipid accumulation with inhibitory effects on other algae is challenging.

Another approach to maintaining contaminant-free algal cultures is to use extremophiles capable of thriving in extreme pH, temperature or salinity conditions, where other organisms cannot (Barnard et al. 2010). To date, a limited number of extremophile algal species are available and used for commercial purposes as opposed to marine and freshwater algae (Mutanda et al. 2011). For example, *Dunaliella salina* (Dunal) Teodoresco is grown in open outdoor raceway systems in a medium with high salinity, while *Spirulina* is grown in a medium with high bicarbonate content and alkaline pH (Jin and Melis 2003, Gantar and Svirčev 2008). High halo-alkaline conditions not only restrict the persistence of contaminants and competing organisms, but may also generate triacylglycerol (TAG) synthesis (Gardner et al. 2011). Furthermore, employing bicarbonate as a carbon source is superior to carbon dioxide injection as it is more soluble and is retained in the culture medium and not lost by outgassing (Chi et al. 2011). The use of carbonates in conjunction with steep alkaline conditions and competent oleaginous microalgae can provide an ideal setting for large-scale production of biofuels and compounds essential to human supplements and drugs (Wensel et al. 2014).

Although application of alkaliphilic or alkali-tolerant microalgae may be practical in mass cultivation, native lipid-producing microalgal strains suitable for these special conditions are ideal. Using native species can be a superior option over non-natives in an
environmental sense to prevent spread of exotics or genetically modified organisms. There are reports of alkaliphilic microalgae from extreme environments, including soda lakes; however, limited efforts have isolated and characterized algae meeting these criteria from freshwater sources, especially from Florida, USA (Selvarajan et al. 2015). The purpose of this study was to exploit the local, lipid-producing, alkali-tolerant / alkaliphilic biodiversity of microalgae, and fill this genetic resource gap. A starting point was to investigate the alkaline tolerant species of our local Lake Okeechobee, Florida.

Lake Okeechobee (26°56′ N, 80°48′ W), located in subtropical south-central Florida, is the largest lake in the southeastern United States. It is a relatively shallow eutrophic lake with a mean depth of 2.7 m (Davis and Marshall 1975, Canfield and Hoyer 1988, James et al. 1995). The lake water levels may undergo wide diel and annual pH fluctuations through man made water control structures and through photosynthesizing phytoplankton (Hagerthey et al. 2011). A high pH in the lake water column can result from photosynthetic withdrawal of carbon dioxide from the water shifting the carbon dioxide-carbonic acid-carbonate equilibrium or the natural limestone benthos. Therefore, we hypothesized that there is a strong possibility for occurrence of native alkali-tolerant microalgae in Lake Okeechobee outflow waters.

In this study, our goal was to isolate and characterize high biomass and lipid producing alkali-tolerant or alkaliphilic microalgae from Lake Okeechobee as novel biological resources for the production of algae-based products. The use of strains with alkaliphilic growth requirements would alleviate the problem of contamination during mass production.
2. Materials and Methods

2.1 Sample collection and enrichment cultures

Surface water grab samples were obtained from different Lake Okeechobee outflows and canals along the Everglades Agricultural Area (EAA) in July 2013. The map of the southern portion of Lake Okeechobee and the 12 sampling sites used in the study (darkened circles) is shown in Supplementary Fig. S1. Water samples were stored in a cooler until transferred to the lab for processing.

Zarrouk’s medium (Vonshak 1993) with an initial pH of 9 and 10 was used for enrichment. Initial enrichment culture flasks for each sampling site consisted of equal volume of 1:1 (v/v) Zarrouk’s medium and water sample. There were three replicate flasks for each sampling site. The flasks were incubated on a rotary shaker at 150 rpm and 25°C under continuous illumination of 60 μmol photons m\(^{-2}\) s\(^{-1}\). After 35 days of cultivation, culture flasks showing visible algal growth were transferred into fresh, pure Zarrouk’s medium supplemented with Gamborg’s B5 solution (1,000×; 1 mL L\(^{-1}\)) and 30 g L\(^{-1}\) of NaSiO\(_3\).

2.2 Selective enrichment of alkali-tolerant isolates

To select for alkali-tolerant isolates, the enrichment cultures were incubated in Erlenmeyer flasks containing modified Zarrouk’s medium at different initial pH values of 9, 10, 11, and 12. The pH of the medium was adjusted using a 10 mol\(^{-1}\) KOH solution. Three replicate flasks were prepared for each pH treatment. Algal suspension from initial enrichment culture was used to inoculate each flask to an initial optical density (OD) of 0.2
at 600 nm. Cultures were incubated on a rotary shaker at 150 rpm and 25°C under a constant light intensity of 60 μmol photons m⁻² s⁻¹ for 35 days.

Growth was assessed using chlorophyll a as an abundance indicator with quantifications carried out every 7 days. A 5 mL aliquot from each Erlenmeyer flask was collected and filtered using a Whatman GF/A (1.6 μm) glass microfiber filter (Whatman Ltd., Buckinghamshire, UK). Sample processing for chlorophyll extraction was adapted from Dere et al. (1998). For this, each filter containing biomass was placed in a 15 mL conical Falcon tube, and 5 mL of 100% acetone was added. Tubes were covered with aluminum foil and stored at 4°C for 24 h. Samples were then centrifuged at 5,000 rpm for 10 min. The supernatant was transferred into a 2 mL cuvette and absorbance was measured at 662 and 646 nm for chlorophyll a and b, respectively using Thermo Scientific Spectronic 200 photospectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Pigment concentration was calculated according to the formulas of Lichtenthaler and Wellburn (1983).

2.3 Isolation and identification of alkali-tolerant strains

Zarrouk’s agar medium adjusted to an initial pH of 9 and 10 were used to isolate alkali-tolerant algae. To obtain pure cultures of individual algal isolates, a 1 mL aliquot of the enrichment culture from each of the pH 9 and 10 treatment flasks was plated on corresponding Zarrouk’s agar (15 g L⁻¹) plates. The plates were incubated for three weeks at 25°C under continuous light (40 μmol photons m⁻² s⁻¹). Individual algal colonies from the alkaline selective agar plates were then streaked onto another plate containing agar medium amended with azithromycin, kanamycin, and streptomycin (10 μg mL⁻¹ each) to
render isolates axenic. Clonal isolates were then transferred to fresh Zarrouk’s medium having a corresponding initial pH and maintained at 25°C.

Genus-level taxonomic identification was based on morphology and carried out using algae identification guides (Lange-Bertalot 2001, Bellinger and Sigee 2015, Wehr et al. 2015). Microscopy of algal isolates was carried out on an Olympus BX51 microscope with attached camera (Olympus DP70) using the corresponding DP controller and manager software applications (Olympus Optical Co. Ltd., Hamburg, Germany). Identification of more complex taxa was carried out using scanning electron microscopy (SEM) detailed below.

2.4 Scanning electron microscopy

Since Fistulifera sp. 154-3 was selected for further study, a more detailed morphological analysis of this strain was performed using SEM (JSM-5900L V; JEOL, Tokyo, Japan). The samples for SEM were prepared using the modified methods of hot hydrogen peroxide (H₂O₂) (Taylor et al. 2007). For this, 10 mL of culture was pipetted into a 15 mL Falcon tube and centrifuged at 3,000 ×g for 15 min at 25°C and the supernatant was removed. The cells were centrifuged and washed twice with 15 mL of sterile BG11 medium. The washed cells were resuspended in 1 mL of deionized water, transferred into a glass beaker and then 10 mL of hydrogen peroxide (H₂O₂) was added (30-35%). The pellet suspended in H₂O₂ was heated to 90°C in a hot water bath for four hours. Once the solution appeared clear, three drops of 50% hydrogen chloride (HCl) were added and the solution was then transferred into a glass centrifuge tube and washed with sterile deionized H₂O three times. Two hundred microliters of washed biomass was pipetted onto a glass
slide and allowed to dry completely. The slide was fixed onto an aluminum stub and gold coated (sputter coater) for SEM imaging.

Alternatively, the samples were prepared using critical point drying (Samdri-PVT-3D; Tousimis, Rockland, MD, USA). Fifty milliliters of culture was pipetted into a 50 mL glass centrifuge tube and centrifuged at 300 ×g for 10 min. The supernatant was removed and replaced with 50 mL of sterile BG11 to remove medium salts and prevent cell lysis. Next, the samples were centrifuged at 300 ×g for 10 min, the supernatant removed, and 20 mL of 2% paraformaldehyde in 0.1 M phosphate buffer was added and incubated at room temperature for 2 h. After incubation, samples were centrifuged at 300 ×g for 10 min and the fixative was removed. Algae cells were then washed with 20 mL of sterile deionized H₂O and cells were allowed to settle. Once settled, 1 mL of cells was pipetted onto a 0.2 μm filter (25 mm; Gelman, Ann Arbor, MI, USA), filtered on low, and then immediately transferred into 30% ethyl alcohol (EtOH). Filters were then transferred sequentially into 50, 70, 90, and 100% ethyl alcohol, allowing 10 min incubation time for each dehydration step. Filters were placed in the critical point dryer (34°C, 1,350 psi) and then gold-coated for SEM imaging.

2.5 Molecular phylogenetic analyses

A 1 mL aliquot of axenic exponential phase cells of Fistulifera sp. 154-3 was transferred into a sterile Eppendorf tube and centrifuged. DNA was extracted using a DNeasy PowerPlant Pro kit (Qiagen, Carlsbad, CA, USA). The nuclear-encoded, small ribosomal subunit (18S rRNA) gene was amplified with polymerase chain reaction (PCR) using GoTaq Green Master Mix (Promega, Madison, WI, USA) with ss3 and ss5 primers
(Rowan and Powers 1991, Matsumoto et al. 2014). To fortify the sequence annotation, internal primers were designed and used including the forward primer fistF: 5′-GGTCCTATTTTGTGTTTGGCG-3′ and the reverse primer fistR: 5′-CGCAAAACCAACAAATAGGACC-3′. The PCR reaction was carried out on an Eppendorf Master Cycler (Nexus GX2; Eppendorf, Hamburg, Germany) using the conditions of 95°C for 45 s, then 35 cycles at 50°C for 45 s, and extension with 72°C for 2 min. The PCR product was visualized on an agarose gel (1%) and then Sanger sequenced by Eurofins Genomics (Louisville, KY, USA). The gene sequence for *Fistulifera* sp. 154-3 is deposited in GenBank (National Center for Biotechnology Information, NCBI) under the accession number KY982278.

BLAST (Basic Local Alignment Search Tool, NCBI) was used for locating strains similar to the 18S rRNA of *Fistulifera* sp. 154-3. Thirty-four diatom sequences were obtained and aligned using MUSCLE (SeaView v4.4) (Edgar 2004), and then manually annotated based on conserved regions. A total of 1,698 bps of the 18S rRNA gene were used from a total of 34 taxa including *Navicula lanceolata* (AY485484) and *Navicula reinhardti* (AM501976) as the outgroup. Phylogenetic trees were constructed using Bayesian inferences (BI) and maximum likelihood (ML) with MrBayes (v3.3.6) and RAxML (7.2.7), through the CIPRES network (v.3.1) (Guindon and Gascuel 2003, Miller et al. 2010). The ML analysis was carried out using the GTR + I + G model, based on jModelTest, assuming heterogeneous substitution rates and gamma substitution of variable sites (proportion of invariable sites [pINV] = 0.390, shape parameter [α] = 0.420, number of rate categories = 4); bootstrap resampling on 1,000 replicates. The BI analysis was conducted using MrBayes 3.6 (Ronquist and Huelsenbeck 2003). Four Metropolis-coupled
Markov chain Monte Carlo (one cold and three heated) were run for $5 \times 10^6$ generations and the first 25% were discarded as burn-in and the following data sets were sampled with a frequency of every 100 generations.

2.6 Screening of strains for lipid accumulation

To determine which of the three isolates accumulated large quantities of lipids, Nile red fluorescence dye (ACROS Organics, Morris Plains, NJ, USA) was used to stain algal cells, both for visualizing lipid droplets within algal cells and for estimating lipid content (Chen et al. 2009). Lipid visualization and quantification were performed by staining algae cells with Nile red and diluting 1 mL of culture to 0.1 OD$_{600}$ and adding dimethyl sulfoxide (20%) and Nile red (1.5 μg mL$^{-1}$) and incubating in the dark for 15 min. Samples were screened in 96-well plates, for autofluorescence and lipid fluorescence using a plate reader (Synergy HTX; Bio Tek, Winooski, VT, USA) and corresponding Gen 5 Analysis software (v3.0) at excitation and emission wavelengths of 530 and 575 nm, respectively. Fluorescence responses of algae were then translated into lipid concentrations using a Triolein lipid standard curve.

2.7 Effect of pH on biomass and lipid accumulation of isolated algae

Once obtained in axenic culture, *Fistulifera* sp. 154-3 was inoculated into Zarrouk’s medium, while *Chloroidium* sp. 154-1, and *Chlorella* sp. 154-2, which did not grow in Zarrouk’s medium, were inoculated into BG11 medium supplemented with 16.8 g L$^{-1}$ of NaHCO$_3$. Biomass and lipid productivity of *Chloroidium* sp. 154-1, *Chlorella* sp. 154-2, and *Fistulifera* sp. 154-3 were evaluated at pH 7-11 (increments of 0.5). Individual algal
species were grown in 150 mL flasks with four replicates each. Aliquots of 1 mL were taken every 5th day for 20 days for both biomass and lipid measurements. Biomass productivity was assessed by filtering 1 mL of culture onto pre-weighted glass microfiber filters (1.2 μm, Whatman GF/C). Filters were dried to constant weight in an oven at 60°C. Lipid content was assessed using the Nile red method and converted lipid value using a Triolein lipid standard curve. Lipid productivity was calculated by multiplying biomass productivity and the lipid content of algae as suggested by Griffiths and Harrison (2009).

2.8 Growth rate, lipid gravimetric, and compositional analyses

The strain that demonstrated highest lipid productivity (Fistulifera sp. 154-3) was selected for specific growth rate and doubling time quantification by inoculating the alga in pH 10 modified Zarrouk’s medium. Biomass, OD (440 nm), and Nile red lipid quantifications were carried out every two days for 23 days, as previously described.

To compare the Nile red method with gravimetric lipid analyses and determine the fatty acid composition of algal biomass, our diatom strain was cultured in triplicate 3 L flasks and harvested on day 10 (exponential phase) by centrifugation at 2,000 × g (GH 3.7; Beckman Coulter, Indianapolis, IN, USA) and lyophilized. For lipid extraction, 1 g of dry algal biomass was placed in glass centrifuge tubes and a modified method by Axelsson and Gentili (2014) was used to extract total lipids in triplicate. Total lipid extract was analyzed to characterize the fatty acid methyl esters (FAMEs). FAMEs analyses were conducted at Gorge Analytical (Gorge Analytical, LLC., Hood River, OR, USA) according to the AOCS Official Method Ce 1-62 (3) (American Oils Chemists Society 2005). Briefly, FAMEs were prepared by transesterification using methanolic hydrochloric acid with an internal
standard (tripentadecanoin; Sigma-Aldrich, St. Louis, MO, USA) and then analyzed with a standard mixture of C₈-C₂₄ FAMEs (Sigma-Aldrich) using gas chromatography with a flame ionization detector (GC-FID) (Agilent Technologies, Santa Clara, CA, USA).

2.9 Statistical analysis

For the selective enrichment of alkali-tolerant isolates, a total of two samples were used with three replicates each at different pH 9-12 and the significant differences between samples at varying pH were determined using a repeated measure, one-way analysis of variance (ANOVA) (Stemmler et al. 2016). Significant differences in biomass and lipid accumulation between the different isolated algae with four replicates were also determined using a one-way ANOVA. A one-way ANOVA was used to determine the possible effect of varying pH 7-12 on biomass and lipid accumulation of Fistulifera sp. 154-3. A one-way ANOVA was also used to determine significant differences between the mean gravimetric lipid weight and the lipid concentration determined using Nile red. All significant differences between treatment means for each variable were compared using a Tukey post-hoc analysis at p < 0.05. All statistical analyses were performed using SPSS software package ver. 22.0 for Windows (IBM Corp., Armonk, NY, USA).
3. RESULTS AND DISCUSSION

3.1 Initial enrichment and isolation

The primary goal of this work was to isolate alkali-tolerant or alkaliophilic algal strains with lipid accumulating properties that could potentially be used for production of biofuel and/or nutraceutical compounds. Using algal strains with extremophile properties for mass cultivation can circumvent contamination in both outdoor and indoor cultivation schemes (Chi et al. 2011). In this work, we used surface water samples from canals coming directly from Lake Okeechobee (FL), whose pH ranges from slightly alkaline to strongly alkaline depending on spatiotemporal factors (Canfield and Hoyer 1988, James et al. 1995). This characteristic of lake water led us to hypothesize that alkali-tolerant strains of algae could be isolated from these samples.

A total of 12 surface water samples were collected from different outflow sites located near Lake Okeechobee as potential sources of alkali-tolerant microalgae. Presumptive alkali-tolerant microalgae were isolated using an alkaline selective enrichment approach. Out of those 12 samples, only site S-308 showed visible growth in two of the replicate cultures (flasks A and B) at pH 9 and 10, respectively (Supplementary Fig. S2). Further selection of cultures from the initial enrichment flasks obtained by using full strength Zarrouk’s medium is shown in Supplementary Fig. S2. In this selective enrichment experiment, only cultures with an initial pH of 9 and 10 had an increase in chlorophyll a content until the end of the cultivation (day 35). Growth of the enrichment cultures from culture flasks A was significantly higher in pH 9 and 10 in comparison to both cultures of flasks A and B inoculated in pH 11 and 12 (p < 0.001 α = 0.05). Enrichment cultures from flasks A were significantly higher in chlorophyll a content than the culture
flasks B inoculated in both pH 9 and 10 (p < 0.001 α = 0.05). There was no significant difference in chlorophyll \(a\) content between the culture flasks A inoculated at both pH 9 and 10 (p = 0.996, \(\alpha = 0.05\)). Chlorophyll content in sample flasks A inoculated in pH 9 and 10 were significantly and consistently higher throughout the 35-day incubation period than flasks B in pH 9, 10, and 12, and as a result, was used for subsequent isolation of algae. In contrast, the cultures with initial pH of 11 and 12 showed some growth initially but died on week 3 and were voided for algal isolations.

Three alkali-tolerant / alkaliphilic strains of microalgae were isolated from the enrichment cultures. Those strains were identified based on morphological features as the chlorophytes *Chloroidium* (strain 154-1) and *Chlorella* (strain 154-2), and a pennate diatom *Fistulifera* (strain 154-3). *Chloroidium* sp. 154-1 and *Chlorella* sp. 154-2 were isolated from Zarrouk’s agar pH 9 plates, while *Fistulifera* sp. 154-3 was isolated from a Zarrouk’s agar pH 10 plate. When stained with Nile red, a stain often used for lipid quantification (Han et al. 2011), *Chloroidium* sp. and *Chlorella* sp. did not show significant accumulation of lipids, while *Fistulifera* sp. accumulated considerable amounts of lipids within the cells (Fig. 1). Thus, *Fistulifera* sp. 154-3 was chosen for lipid analysis.
Fig. 1. Bright field and Nile red fluorescence, respectively, of Chloroidium sp. 154-1 (A & B), Chlorella sp. 154-2 (C & D), and Fistulifera sp. 154-3 (E & F). Yellow fluorescence is indicative of lipid bodies; red fluorescence indicates chlorophyll autofluorescence of cells (×1,000). Scale bars represent: A-F, 10 μm.
3.2 Biomass and lipid accumulation of algal isolates under increasing pH

Biomass production and lipid accumulation of the isolated microalgae varied depending on the strain and pH of the medium (Supplementary Table S1). Over the 20-day cultivation period, *Chloroidium* sp. 154-1 had the highest mean biomass (2.64 g L\(^{-1}\)) at pH 8.5. The highest mean lipid content for *Chloroidium* sp. 154-1 occurred at pH 9, with up to 4.51% dry biomass. The highest mean biomass and lipid content for *Chlorella* sp. 154-2 was observed in pH 9 with 2.95 g L\(^{-1}\), and pH 10.5 with 5.86% dry biomass. The highest mean biomass and lipid content for *Fistulifera* sp. 154-3 was observed at pH 10 with 2.68 g L\(^{-1}\), and pH 9 with up to 8.78% dry biomass over the 20-day incubation period.

During the 20-day sampling period, the highest lipid accumulation among algal strains tested was recorded for *Fistulifera* sp. 154-3 on day 10. The biomass and lipid percentage data from only day 10 are therefore presented for all three algal strains, the data from the rest of the sampling days (5, 15, and 20) are provided in Supplementary Table S1. The dry biomass yield and lipid content for all three strains, *Chloroidium* sp. 154-1, *Chlorella* 154-2, and *Fistulifera* sp. 154-3 on the tenth day of cultivation, are shown in Fig. 2. A maximum dry biomass of 3.05 g L\(^{-1}\) was recorded for *Chloroidium* sp. 154-1 and 2.93 g L\(^{-1}\) for *Chlorella* sp. 154-2 and the lipid content in those two strains was never >11% across the pH levels tested over 20 days of cultivation. The highest dry biomass (3.1 g L\(^{-1}\)) of *Chloroidium* sp. 154-1 was observed in pH 8.5, while the highest lipid accumulation (9.34%) was found at pH 11 (Fig. 2A). In *Chlorella* sp. 154-2, the highest dry biomass of 2.93 g L\(^{-1}\) occurred at pH 9, while the highest lipid accumulation (3.67%) resulted in the pH 10.5 treatment (Fig. 2B). The mean values for lipid content of *Chloroidium* sp. 154-1 at pH 11 and *Chlorella* sp. 154-2 at pH 10.5 were highly variable, as most of the replicates
ceased to grow and flasks contained mostly dead cell debris (Supplementary Fig. S3). In *Fistulifera* sp. 154-3, maximal lipid accumulations of 16, 17, and 19% were recorded at pH 9.5, 8.5, and 9, respectively.
Fig. 2. Dry biomass (g L\(^{-1}\) dry weight) and lipid percentages (g g\(^{-1}\) dry biomass) of *Chloroidium* sp. 154-1 (A), *Chlorella* sp. 154-2 (B), and *Fistulifera* sp. 154-3 (C), on day 10 of cultivation in varying pH 7-11. Data represents the mean of four replicates.
**Chloroidium** sp. 154-1 had the highest lipid concentration on day 5 of incubation with up to 11.03% of dry biomass. The lipid content gradually decreased for the remaining incubation period. The highest mean lipid content occurred in pH 9.5 with up to 4.51% of dry biomass. Using pH 8.0 as a control, the biomass of **Chloroidium** sp. 154-1 was significantly lower than biomass found at pH 8.5, 9.5, 10, and 10.5 (p = 0.001, p = 0.014, p = 0.003, p < 0.001 α = 0.05). The biomass at pH 8.5 was consistently higher than all other pH levels on days 10, 15, and 20. The biomass of **Chloroidium** sp. 154-1 was significantly higher at pH 8.5 than 7, 7.5, 8, and 11 (p = 0.005, p = 0.011, p = 0.001, p < 0.001, α = 0.05) on day 10.

For **Chlorella** sp. 154-2, using pH 8 as a control on day 10 of cultivation, the only significantly higher biomass occurred at pH 10 (p = 0.041, α = 0.05). The biomass accumulation of **Chlorella** sp. 154-2 in pH 9 was significantly higher than the pH levels of 7.5, 10, 10.5, and 11 (p = 0.004, p = 0.000, p = 0.000, p = 0.001, α = 0.05). Conversely, the lipid content at pH 10.5 was significantly higher than the lipid content observed at pH 7, 8, 8.5, 9, and 9.5 (p = 0.004, p = 0.001, p = 0.003, p = 0.001, p = 0.001, α = 0.05).

On day 10 of incubation, the biomass of **Fistulifera** sp. 154-3 was significantly lower in pH 8 than in pH 9.5 and 10 (p = 0.007, p = 0.000, α = 0.05). The biomass of **Fistulifera** sp. 154-3 at pH 10 was significantly higher than pH levels of 7, 7.5, 8, 8.5, 9, 10.5, and 11 (p < 0.007, α = 0.05). There were no significant differences between the biomass of **Fistulifera** sp. 154-3 at pH 9.5 and 10 (p = 0.366, α = 0.05). The lipid content of **Fistulifera** sp. 154-3 found in pH 8 was significantly lower than that observed in pH 8.5, 9, 9.5, and 10 (p = 0.000, p = 0.000, p = 0.000, p = 0.011, α = 0.05). The lipid content within **Fistulifera** sp. 154-3 was significantly higher at pH 9 than in pH 7, 7.5, 8, 10.5, and
11 (p < 0.001 α = 0.05). *Fistulifera* sp. 154-3 cultured between pH 8.5 and 9.5 showed 3 to 5 times higher lipid content than *Chloroidium* sp. 154-1 and *Chlorella* 154-2 (Fig. 2C).

When comparing the biomass and lipid content of the three isolates on day 10 of cultivation, the biomass of *Chloroidium* sp. 154-1 and *Chlorella* sp. 154-2 were both consistently higher than *Fistulifera* sp. 154-3 in all pH levels tested except 11. Conversely, the lipid content of *Fistulifera* sp. 154-3 was higher than both *Chloroidium* sp. 154-1 and *Chlorella* sp. 154-2 in all pH levels tested. Since *Fistulifera* sp. 154-3 displayed higher concentrations of lipids, this strain was chosen for further productivity calculations and fatty acid determinations.

The biomass and lipid productivity of *Fistulifera* sp. 154-3 were evaluated at pH 7-11 in increments of 0.5, in Zarrouk’s medium. The strain was able to grow over a wide range of pH (Supplementary Fig. S4A); the highest biomass productivity (0.415 g L^{-1} d^{-1}) was recorded after five days of cultivation at pH 8.5. This biomass productivity on day 5 was almost 2-fold significantly higher than at pH 7, 9, 9.5, 10.5, and 11 (p =0.048, α = 0.05). Thereafter, the highest biomass productivity was recorded at pH 10 on the day 10, 15, and 20 of cultivation with resulting productivities of 0.233, 0.240, and 0.169 g L^{-1} d^{-1}, respectively (Fig. Supplementary Fig. S4a). The biomass productivities at pH 10 on day 10 and day 15 were significantly higher than all the rest of the pH treatments (p =0.007, α = 0.05). The difference in medium pH values resulted in substantial variation in the lipid productivity throughout cultivation (Supplementary Fig. S4B). The highest mean lipid productivity among the pH treatments was found on day 10 of cultivation. On the day 10 of cultivation, the lipid productivities of *Fistulifera* sp. 154-3 ranged between 8.08 and 39.95 mg L^{-1} d^{-1}. *Fistulifera* sp. 154-3 cells showed higher lipid productivity at pH 8.5, 9,
9.5, and 10, than at pH 7, 7.5, 10.5, and 11. After quantification of lipid and biomass productivity, specific growth and doubling time of the diatom were investigated.

A separate growth run demonstrated that the average specific growth rate of *Fistulifera* sp. 154-3 was $\mu = 0.832 \text{ d}^{-1}$ with a corresponding doubling time of 20 h. The specific growth rate and doubling time is comparable to most diatoms previously described (Griffiths and Harrison 2009). Additionally, when inoculated in increasing pH from 7 to 11, all of the initial pH levels were naturally altered until reaching pH of 10.2-10.3 after a week of incubation and remained at that level thereafter (Supplementary Fig. S5). The pH changed drastically after one day of incubation for all treatments except that of pH 10, which remained constant. As a result, *Fistulifera* sp. 154-3 is a diatom capable of manipulating the medium pH through photosynthesis to suitable alkalinity levels for proper growth. A naturally alkalizing diatom can be an ideal tool in mass cultivation where a constant pH is kept without artificial buffers.

From Lake Okeechobee, we have isolated two algal strains that could be characterized as alkali-tolerant and one alkaliphilic. The S-308 site water sample from which these strains were isolated had a pH of 8.2 (± 0.2). *Chloroidium* sp. 154-1 and *Chlorella* sp. 154-2 (Fig. 2) with optimal growth $\geq$ pH 8-9 were designated as biomass-accumulating alkali-tolerant algae, according to Jones et al. (1994) and Gimmler and Degenhard (2001). The diatom *Fistulifera* sp. 154-3 (Figs 1, 2, Supplementary Figs S4 & S5) with an optimal growth from pH 8.5 up to 10.3, and highest lipid productivity at pH 10, is designated as alkaliphilic (Kroll 1990, Gimmler and Degenhard 2001). Since *Fistulifera* sp. 154-3 demonstrated high alkaline tolerance and moderate biomass and lipid productivities, it was chosen for further characterizations.
3.3 SEM confirms genus *Fistulifera*

SEM microphotographs of *Fistulifera* are depicted in Supplementary Fig. S6. The individual cells of *Fistulifera* sp. 154-3 are below 10 μm in size (Fig. 1). Frustules of *Fistulifera* sp. 154-3 have symmetrical valves up to 7 μm in length; substituting valve length for raphe length since the genus is lightly silicified resulting in a reduced valve margin. The genus *Fistulifera* is characterized by light silicification of the frustule, a unique raised raphe sternum, and fistula opening in the central region of the valve (Supplementary Fig. S6E-G). A few species have been characterized within this genus including *Fistulifera solaris* S. Mayama, M. Matsumoto, K. Nemoto & T. Tanaka, *F. saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, *F. iranensis* (Hustedt) Lange-Bertalot, and *F. pelliculosa* (Kützing) Lange-Bertalot (Zgrundo et al. 2013, Matsumoto et al. 2014, Guiry and Guiry 2018). *Fistulifera saprophila* is found in eutrophic waters, while *F. pelliculosa* is restricted to oligotrophic waters. *Fistulifera pelliculosa* and *F. saprophila* both secrete mucilage when cells cease to replicate (Lewin 1955, Zgrundo et al. 2013). *Fistulifera solaris* differs since it is a marine species and is slightly larger (Matsumoto et al. 2014). *Fistulifera* sp. 154-3 can be distinguished from the other previously described *Fistulifera* species by its lack of mucilage production throughout its growth cycle and has a thinner girdle band (Supplementary Fig. S6B). Interestingly, *Fistulifera* sp. 154-3 also forms colonies of 2-10 cells, as documented for *F. saprophila* (Zgrundo et al. 2013).

The ubiquitous genus *Fistulifera* is comprised of species that occur in fresh, brackish or saline waters, at varying degrees of pollution, pH, and salt concentration. To date, the species that are well characterized within this genus include: *Fistulifera solaris*, *F. iranensis*, *F. saprophila*, and *F. pelliculosa*. Studies on this genus have focused on the
strain *F. solaris* JPCC DA0580, which has high lipid accumulation (40-60%), but limited to growth in artificial seawater (Matsumoto et al. 2010, Sato et al. 2014, Muto et al. 2015, Tanaka et al. 2015) with possible growth in brackish water (Satoh et al. 2013).

Conversely, *Fistulifera* sp. 154-3 thrives in modified Zarrouk’s medium, adjusted at pH 10 and 1.4% salinity (Supplementary Fig. S3C). *Fistulifera* sp. 154-3 accumulates the highest levels of lipids on day 10 at pH 9 and when biomass is taken into account, lipid productivities at pH 8.5-10 are superior to the other treatments (Supplementary Fig. S4A & S4B). Additionally, the diatom is capable of naturally manipulating the media to suitable pH levels near 10.2 (Supplementary Fig. S5). *Fistulifera* sp. 154-3 demonstrates how adaptation of this genus to varying physicochemical conditions, from oligotrophic, eutrophic, to increasing halo-alkaline conditions, could be conducive to minimizing invading organisms during cultivation. Thus, we identified *Fistulifera* sp. 154-3 as an alkaliophilic alga that could facilitate the mass cultivation of algae in both indoors and outdoors schemes.

In using *Fistulifera* sp. 154-3 for outdoor cultivation, sodium bicarbonate becomes an ideal component in cultivation. Sodium bicarbonate is a superior carbon source for algal cultivation since the natural salt concentration increases the pH of the medium. Atmospheric carbon can also be captured in the form of bicarbonate at a fraction of the cost, and this carbon can be stored and transported without significant loss to the atmosphere. Additionally, using bicarbonate and a high pH cultivation environment leads to minimal carbon loss by outgassing (Chi et al. 2011). As *Fistulifera* sp. 154-3 uses up the bicarbonate in solution, the medium becomes a carbon sink and pulls CO₂ from the air to maintain the bicarbonate/ carbonate buffer system and provide the diatom with a carbon
source. Using sodium bicarbonate to increase pH values of the culture medium can be a good strategy, not only in preventing culture contamination, but also as a factor stimulating lipid accumulation (Gardner et al. 2011).

3.4 Molecular phylogenetic analyses

A more detailed taxonomic identification of *Fistulifera* sp. 154-3 was performed using the 18S rRNA gene (Fig. 3). The phylogenetic analysis shows that the genus *Fistulifera* is a well-supported (bootstrap [BS], 100%; posterior probability [PB], 1.0) monophyletic clade (box in Fig. 3). In the analysis, several sequences of *Fistulifera pelliculosa* including AJ544657 and EU260468 did not fall within the *Fistulifera* clade, but with *Mayamaea* sp. (KY863471). However, the isolate *Fistulifera* sp. 154-3 formed a well-supported distinct subclade that included *Navicula* sp. JB12 (KF791556) and JB16 (KF791555) (BS, 63%; PB, 0.93). The subclade that contains *Fistulifera* sp. 154-3 and the *Navicula* sp. strains are isolated from high alkaline waters and soils, respectively, whereas the remaining *Fistulifera* strains are located in separate subclades with related saline and freshwater strains; which delimited into strongly supported branches for *F. saprophila* (BS, 97%; PB, 1.0) and *F. pelliculosa* (BS, 61%; PB, 0.87) as well. The phylogenetic distance of *Fistulifera* sp. 154-3 suggests that it might represent a novel species to science, but a more detailed characterization of this strain is necessary for a proper taxonomic description.
Fig. 3. Bayesian inferences (BI) and maximum likelihood (ML) phylogenetic tree showing phylogenetic relationships among *Fistulifera* sp. 154-3 and the taxa sequences analyzed based on 18S rRNA gene data (34 taxa; 1,698 bp). ML and BI analysis bootstrap values and posterior probabilities (>50%, 0.5) are indicated at each node.

Lipid analyses

To compare the Nile red method with traditional gravimetric techniques, *Fistulifera* sp. 154-3 was cultured until the exponential phase and analyzed in triplicate. The mean lipid content of the diatom was 31.0 and 23.2% for the Nile red and gravimetric methods, respectively (Supplementary Fig. S7). There was no significant difference in the mean lipid content determined with either detection method ($p = 0.277, \alpha = 0.05$).
Lipid analyses of *Fistulifera* sp. 154-3 revealed a profile predominantly abundant in monounsaturated followed by saturated and then polyunsaturated fatty acids (Table 1). Monounsaturated carbon methyl chains include 52% PA (C16:1c), 1.3% myristoleic acid (C14:1c), 5% co-elution of oleic / elaidic acid (C18:1c; C18:1t), and 0.74% cis-10-heptadecenoic acid (C17:1). In terms of saturated compounds, *Fistulifera* sp. 154-3 can produce around 17% palmitic acid (C16:0), 5% myristic acid (C14:0), and 0.41% stearic acid (C18:0). Polyunsaturated compounds include about 16% cis-5,8,11,14,17 eicosapentaenoic acid (C20:5n3), 1.2% co-elution of linoleic / linolelaidic acid (C18:2c; C18:2t), 0.34% arachidonic acid (C20:4n6), and 0.31% of γ-linolenic acid (C18:3n6).

<table>
<thead>
<tr>
<th>Fatty acid (methyl ester)</th>
<th>Mass (%) methyl ester</th>
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<tr>
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<tr>
<td>cis-10-Hepadecanoic acid</td>
<td>0.74</td>
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<tr>
<td>Stearic acid</td>
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<tr>
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<tr>
<td>Cis-5,8,11,14,17 eicosapentaenoic acid</td>
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</tr>
</tbody>
</table>

*Table 1.* Fatty acid profile\(^a\) of *Fistulifera* sp. 154-3 lipid obtained at pH 10 in modified Zarrouk’s medium after 10 days of cultivation

\(^a\)Fatty acid name (carbon number: number of double bonds); fatty acid profiles of lipid was analyzed according to AOCS Official Method Ce 1-62 (3) (American Oils Chemists Society 2005).
Most of the fatty acids accumulated by our *Fistulifera* sp. 154-3 are represented by PA (C16:1c), palmitic acid (C16:0), and Cis-5,8,11,14,17 eicosapentaenoic acid (C20:5n3) (Table 1). The lipid profile of *Fistulifera* sp. 154-3 has implications for both medicinal and environmental sectors. This diatom produces fatty acids (FAs) ideal for fuel substitutes as it produces many long, saturated carbon chains, such as palmitic acid, and polyunsaturated carbon compounds. Additionally, PA has an industrial value for polyethylene production and biofuels (Nguyen et al. 2015).

An additional key potential commercial application of *Fistulifera* sp. 154-3 lies in the production of omega fatty acids as a human supplement, including PA (ω-7) and eicosapentaenoic acid (ω-3). Omega-7 fatty acids can be extracted from *Fistulifera* sp. 154-3 without the risk of fish-associated toxic contaminants and avoid competition with the traditional agricultural sources of these omega fatty acids including macadamia nuts (*Macadamia integrifolia*) and sea buckthorn berries (*Hippophae rhamnoides*) (Bal et al. 2011, Copat et al. 2011. To date, PA can be sourced from a variety of organisms including yeasts and algae, but restricted to the type of cultivation used. Yeast-based sources have shown to produce only up to 16% palmitoleic acids and limited to fermentative schemes (Kolouchová et al. 2015).

Alternatively, the marine alga *Nannochloropsis salina* Hibberd can accumulate PA between 22 to 30% of its total fatty acids (Ma et al. 2016) with possible increases of up to 35% based on altering cultivation light and temperature regimes (Van Wagenen et al. 2012). Another alga, *Heterococcus* sp., was found to produce 20-55% PA in temperatures of 4-22°C, but is only suitable for cultivation in cold temperate climates (Nelson et al. 2013). In the present study, the alkaliphilic diatom *Fistulifera* sp. 154-3 was able to produce
up to 52% PA when exposed to a warm temperature of 25°C, allowing for cultivation in a subtropical climate.

Our isolate *Fistulifera* sp. 154-3 accumulates around (20-30%) lipids, with a lipid productivity of around 40 mg L\(^{-1}\) d\(^{-1}\), and we believe that by manipulating cultivation regimes, a higher lipid production can be achieved (D’Ippolito et al. 2015). In the future, we aim to optimize the biomass and lipid productivity of *Fistulifera* sp. 154-3 by manipulating the nutrient composition (bicarbonate and B vitamins), salt concentration, temperature, and light intensity during cultivation. Additional morphological and molecular analyses are required to reveal the true phylogenetic placement of our *Fistulifera* isolate.
4. CONCLUSION

High halo-alkaline conditions in algae cultivation may restrict the persistence of contaminants and competing organisms, which can facilitate the scaling-up process. Since there is limited number of extremophilic algae available for cultivation, the purpose of this study was to explore Florida native, lipid-producing, alkali-tolerant / alkaliphilic microalgae diversity. Bioprospection and initial selection for extremophilic algae from Lake Okeechobee resulted with the isolation of three algal strains: two alkali-tolerant isolates and one alkaliphilic isolate. Using Nile red to determine intracellular lipid concentrations revealed an alkaliphilic diatom with high lipid productivity around 40 mg L$^{-1}$ d$^{-1}$. Morphology and 18S rRNA gene sequence phylogeny showed the diatom belonged to a genus of small raphid diatoms, *Fistulifera*. Gravimetric analyses indicated a diatom where 30% of its biomass are lipids, and gas chromatography showed a fatty acid profile to mainly consist of palmitoleic, palmitic, and eicosatetraenoic fatty acids. In conclusion, the isolated strain *Fistulifera* sp. 154-3 is an alkaliphilic diatom with potential use in mass cultivation systems to produce high-value lipid compounds, such as $\omega$-7, $\omega$-3, and palmitic acid. Omega-7 and $\omega$-3 can be used for nutraceutical production, and palmitic acid can be used as a feedstock for biodiesel.
ACKNOWLEDGEMENTS

NDR would like to acknowledge funding by USDA-NIFA National Needs Fellows [2011-38420-20053]. DEB would like to acknowledge partial funding by USDA-NIFA-HSI Biofuels Science Education at FIU [2010-02067]. HDL would like to acknowledge the USDA NIFA Hatch project FLA-FTL-005697. We would also like to thank M. Barbosa for constructing the map of Lake Okeechobee (Supplementary Fig. S1) using ArcGIS.

SUPPLEMENTARY MATERIALS
Supplementary Table S1. Biomass (g L⁻¹) and lipid content (% dry biomass) of *Chloroidium* sp. 154-1, *Chlorella* sp. 154-2, and *Fistulifera* sp. 154-3 inoculated in increasing pH 7-11 (increments of 0.5) over 20 days of incubation (https://e-algae.org).

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**Supplementary Fig. S1.** Sampling points along the Everglades Agricultural Area (EAA) (https://e-algae.org).
Supplementary Fig. S2. Growth based on chlorophyll content of enrichment cultures (flasks A and B) at pH 9 (A), pH 10 (B), pH 11 (C), and pH 12 (D) in modified Zarrouk’s medium over 35 days. Growth is expressed as chlorophyll a content per mL of medium (μg mL⁻¹). Data represents the mean of three replicates (https://e-algae.org).
Supplementary Fig. S3. Culture flasks containing *Chloroidium* sp. 154-1 (A), *Chlorella* sp. 154-2 (B), and *Fistulifera* sp. 154-3 (C), inoculated in medium of varying pH 7-11 (increments of 0.5) on day 10 of cultivation. Showing one of four replicates (https://e-algae.org).
Supplementary Fig. S4. Biomass (g L\(^{-1}\) d\(^{-1}\)) (A) and lipid (mg g\(^{-1}\) d\(^{-1}\)) (B) productivity of Fistulifera sp. 154-3 over 20 days cultivation in varying pH 7-11 (increments of 0.5). Data represents the means of four replicates (https://e-algae.org).
Supplementary Fig. S5. Recorded pH of inoculated media with *Fistulifera* sp. 154-3 up to 23 days of incubation. Results are measurements of four replicates, error bars omitted for simplicity (https://e-algae.org).
**Supplementary Fig. S6.** Scanning electron micrographs of *Fistulifera* sp. 154-3 prepared by critical-point drying (A-C) and hot peroxide methods (D-H). Arrowhead depicting cell to cell contact (A), rotated side-view and top view showing thick girdle bands (B & C), top view of valve framed by fimbriate margin with central fistula opening (D), external valve showing raphe opening, curvature, and light silicification (E), internal view of top valve with distinct raphe sternum (arrowhead) (F), external view of valve with characteristic fistula (arrowhead) (G), internal valve view showing striae, protruding raphe sternum, and opening of fistula (arrowhead) (H). Scale bars represent: A-H, 1µm (https://e-algae.org).
Supplementary Fig. 7. Comparison of lipid content (%) within dry algal weight between gravimetric and Nile red lipid quantification methods. Results indicate the mean of triplicates (https://e-algae.org).
REFERENCES


II. CHAPTER 2

*Fistulifera alcalina* sp. nov. (Bacillariophyceae) a New Alkaliphilic Diatom Species

from Lake Okeechobee, Florida (USA)

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*Fistulifera alcalina* sp. nov. (Bacillariophyceae) a New Alkaliphilic Diatom Species from Lake Okeechobee, Florida (USA)

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ABSTRACT

Small and lightly silicified diatoms are often overlooked and underrepresented in diatom diversity studies. In this work, we propose and describe a novel diatom species *(Fistulifera alcalina* sp. nov) isolated from Port Mayaca on Lake Okeechobee, FL (USA). This new diatom species represents a distinct lineage within the genus *Fistulifera*, which includes *F. iranensis* (Hustedt) Lange-Bertalot, *F. saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, *F. pelliculosa* (Kützing) Lange-Bertalot, and *F. solaris* (S. Mayama, M. Matsumoto, K. Nemoto & T. Tanaka.). Morphometric and molecular phylogenetic analyses of the 18S rRNA and *rbcL* genes strongly support *Fistulifera alcalina* as a new species. *Fistulifera alcalina* sp. nov. is cultured in media with moderate salinity and high bicarbonate concentrations in contrast to the other species of *Fistulifera* species which bear ecological preferences for high salinity and eutrophic or oligotrophic waters. This is also the first record of *Fistulifera* in the southeastern United States (Florida, USA).

**Key words:** New species, SEM, lipid production, fistula, Bacillariophyta, alkaline
1. INTRODUCTION

*Fistulifera* is a genus of relatively small (4-13µm) raphid diatoms that require electron microscopy for detailed morphological descriptions. This diatom has lightly silicified frustules with inconspicuous margins and few features discernible using light microscopy (LM). Though small, their global range is broad and includes the Arctic (Michelutti et al. 2007), temperate (Gevrey et al. 2004, Vouilloud et al. 2005), and subtropical (this study, Hustedt 1962) regions. Within these climates, *Fistulifera* has a widespread ecological occurrence, inhabiting fresh (Lewin 1955, Stancheva et al. 2007), brackish (Della Bella et al. 2007), saline and marine (Hustedt 1962, Matsumoto et al. 2014) waters, and terrestrial environments (see *Navicula* JB12 Genbank #KF791556; Qiao & Liu, unpub.). Furthermore, the genus is found in a spectrum of water trophic states including oligotrophic, eutrophic, and even hypereutrophic (Michelutti et al. 2007).

The first record of a species of *Fistulifera* was in 1849, identified as *Synedra minutissima* var. *pelliculosa* Kützing. Afterwards, the description of the genus *Fistulifera* was emended and ascribed to the genus *Navicula* (Lange-Bertalot & Bonik 1976). Lange-Bertalot (1997) later amended this description and erected the genus *Fistulifera*, including the type species *Fistulifera saprophila* (Lange-Bertalot & Bonik) Lange-Bertalot, along with two other species: *F. iranensis* (Hustedt) Lange-Bertalot and *F. pelliculosa* (Brébisson ex Kützing) Lange-Bertalot. Afterwards, the discovery of *F. solaris* (S.Mayama, M.Matsumoto, K.Nemoto & T.Tanaka) expanded the range and distribution of this genus to Japanese marine waters (Matsumoto et al. 2014, Guiry & Guiry 2018).
More recently, Berthold et al. 2019 (submitted) found a unique alkaliphilic diatom, *Fistulifera* sp. 154-3, while bioprospecting Lake Okeechobee for putative alkali-tolerant algae. *Fistulifera* sp. 154-3 was exceptional as it grew in media with high alkalinity (pH 10) that is favorable for overcoming cultivation contamination (Barnard et al. 2010). This study presents an ecological, morphological, and molecular description of *Fistulifera alcalina* sp. nov (*Fistulifera* strain BLCC-F88), a new taxon within a genus that is easily overlooked considering its small size and fragile frustule. Light microscopy (LM) and scanning electron microscopy (SEM) of morphological features along with 18S rRNA and *rbcL* (ribulose-1,5-biphosphate carboxylase oxygenase) molecular phylogeny results indicated that this taxon is distinct, emending the genus *Fistulifera* by including alkaline species. Since this species was unique, *F. alcalina* is described here to fill the genetic gap within the currently established genus. This work also provides the first record of this genus in the southeastern United States (Florida, USA) further expanding both species range and ecological preference of these diatoms.

2. MATERIALS and METHODS

2.1 Algae Isolation and Culture

Water surface and column samples were taken near the Port Mayaca lock of Lake Okeechobee, Florida during summer 2014. Selection for alkaliphilic or alkali-tolerant species included using protocols described in Berthold et al. 2019 (submitted). Single colonies of *F. alcalina* were exposed to antibiotics (streptomycin, penicillin, and neomycin; 300µl ml\(^{-1}\); Gibco, Life Technologies) to yield axenic cultures. *Fistulifera alcalina* was maintained and cultured in Zarrouk’s medium modified by the addition of
NaSiO₃ (30g L⁻¹) and 10M NaOH until pH 10 (~200mM). Cultures were incubated in 125ml flasks exposed to 50 µmol m² s⁻¹ fluorescent light with constant shaking (120rpm).

2.2 Morphometric Analysis

LM images of live and oxidized *F. alcalina* were captured using a Leica DM5500B microscope with differential interference contrast (DIC) and corresponding Leica software (LAS V4.12), while fluorescent images were taken of only live cells using an Amscope FX2334 with corresponding software. A total of 50 live diatom cells were used for the measurements. Diatom terminology was derived from Round et al. (1990) and Cox (1996). To remove organic material, a hot peroxide (30%) method was used to prepare the diatom for SEM analysis, where 1ml of exponential phase *F. alcalina* culture was transferred into an Eppendorf tube, centrifuged, then washed five times with deionized water to remove carbonates. A 300 µL aliquot of cleaned cells was then pipetted into a glass vial and diluted with 5ml of 30% hydrogen peroxide. The glass vial was heated in a hot water bath until the material was completely oxidized. Since *F. alcalina* is an oily alga cultured in high carbonate concentrations, a few drops of 10% hydrochloric acid (HCl) was added to remove residual carbonates and organic fatty material. 100µL of cleaned material was placed on a 0.22µm filter and washed with deionized water over a vacuum to remove all material previously observed to coat and mask cells under SEM. Dried filter disk with the cleaned material was then gold coated using a Leica EM ACE600 sputter coater. Processed material was imaged using a FEI, Apreo SEM (NMNH-SI; Thermo Fisher Scientific) with various detectors (T1, T2, T3). The oxidized material was also used to make a permanent slide with Naphrax, and
subsequently used to measure an additional 50 oxidized cells. The type and isotype material and slides of *F. alcalina* are deposited in the US National Herbarium under the accession numbers USNH D31023 and D31022, respectively (National Museum of Natural History, Smithsonian Institution, Washington, DC USA).

For morphometric analysis, the length, width, striae number and density, areolae density, and distance between areolae of 50 cells under SEM were quantified (ImageJ 1.51s). The length of the raphe sternum was substituted for the length of the valve face because of the weakly silicified valve margin (Matsumoto et al. 2014). Valve width was measured within the area of areolation. Distances between areolae were measured as suggested by Matsumoto et al. (2014), where the distance between centers of terminated areolae and the fifth one was measured. Striae were counted first within a 2.5µm distance and conventionally adjusted to 10µm.

### 2.3 Molecular and Phylogenetic Analyses

To extract DNA, axenic exponential phase diatoms were transferred into an Eppendorf tube and washed three times with deionized water. Total algal genetic material was extracted using a DNeasy Plant Mini kit (Qiagen Inc, USA). The *rbcL* and 18S rRNA genes were selected for amplification using primers displayed in Table 1. Methods for the 18S rRNA gene can be found in Berthold et al. (submitted). The *rbcL* gene was amplified using a GoTaq PCR Core systems kit (Promega, USA) on a ProFlex thermocycler (Applied Biosystems, Life technologies) following Jones et al. (2005), with modifications. Briefly, 10 µL TBE buffer, 10 bovine serum albumin (BSA), 8 µL MgCl₂, 1 µL dNTPs, 0.25 µL GoTaq G2, 1µL template DNA, and 1 µL of each primer were
pipetted into a PCR reaction tube. PCR amplification involved an initial denaturation for 3 min at 94°C and final extension for 10 min at 72°C, and thirty cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. PCR products of all genes were verified on a 1% agarose gel and subsequently sequenced using the Sanger method by Eurofins Genomics (Louisville, KY, USA). The rbcL gene sequence can be found in GenBank (NCBI) under the accession number MN251841.

### TABLE 1. Primers used in this study

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F (Forward) and R (Reverse) primer orientation in relation to rRNA
A total of 1452 base pairs of the rbcL gene sequence were aligned from 24 taxa using MUSCLE (SeaView v4.4) (Edgar 2004), including Navicula lanceolata (AY485484) as outgroup. A total of sites of 1658 base pairs of the 18S rRNA gene were aligned with 44 taxa using Navicula lanceolata (AY485484) and Navicula reinhardii (AM501976) as outgroup. Phylogenetic trees for both genes were constructed using Bayesian inferences (BI) and Maximum likelihood (ML) with MrBayes (v3.3.6) and RAxML (7.2.7), respectively, on the CIPRES network (v.3.1) (Guindon & Gascuel 2003, Miller et al. 2010). Best-fit models were tested using JmodelTest. The ML analyses were carried out using the GTR+I+G model with bootstrap resampling on 1,000 replicates. Bayesian analysis was conducted using MrBayes 3.6 (Ronquist & Huelsenbeck 2003). Two runs of four MCMC chains (three heated and one cold) were run for 1.5 x 10^6 generations, sampling every 100 trees. For each run, the first 25% were discarded as the burn-in phase.
3. RESULTS

*Fistulifera alcalina* Berthold, Frankovich, Gaiser et Laughinghouse IV sp. nov. (Figs 1–17)

**Diagnosis:** SEM and LM is required to morphologically differentiate *Fistulifera alcalina* from similar *Fistulifera* species. *F. alcalina* is most similar to *F. solaris* sharing very small, delicate, lightly silicified frustules with one chloroplast. Using SEM, the central nodule of *F. solaris* is observed with a deep groove providing little distinction with the surrounding raphe and raphe-sternum (see fig. 15 in Matsumoto et al. 2014). In contrast, the central nodule of *F. alcalina* is prominently raised from the internal valve surface with only a shallow groove (Fig 16). Stria densities also differ between the two species (i.e., 60-72 and 54-59 in 10 µm for *F. alcalina* and *F. solaris*, respectively). *F. alcalina* can also be differentiated from *F. saprophila* by chloroplast number: 2 parietal plastids in *F. saprophila* versus one plate-like plastid in *F. alcalina*. *F. alcalina* can be differentiated from *F. iranensis* by differing valve lengths (4-6 µm versus 10-11 µm, respectively) and valve outline (elliptical versus linear elliptical, respectively). Additional morphological and ecological differences and similarities between the species are summarized in Table 2.
3.1 Description

Live observations (Figs 3–12): Cells solitary (Figs 3, 11), sometimes forming chains of up to 8 during division (Figs 4–10, 12). Frustules rectangular to ovoid with broadly rounded ends in girdle view (Figs 4–10). Single H-shaped plate-like chloroplast with two lobes connected by isthmus; lobes diagonally opposed at the apices, extending onto girdle (Fig. 12). Large lipid bodies (2 depicted) towards apices (Fig 11, fluorescence microscopy). Valves elliptical (Fig 3).

Light microscopy (cleaned material, Figs 1–2): Valves lightly silicified, outline barely discernible with prominent thickened raphe-sternum, central and polar terminal nodules (Figs 1–2, phase-contrast). Striae indiscernible. Valve dimensions: length 6.4–7.8 µm (mean 7.2 µm; sd 0.37), width 3.9–5.0 µm (mean 4.4 µm; sd 0.25).

Scanning electron microscopy (Figs 13–17): External view: Valve face flat (Fig 14). Valve margin eroded and cingulum not observed following cleaning process due to fragile nature of frustules; valve mantle artefactually fimbriate (Figs 13–15). Raphe branches slightly curved towards primary side of valve and surrounded by narrow axial area flush with valve face (Fig 14). Central raphe ends straight, simple, somewhat distantly spaced (Fig 14). Terminal raphe fissures deflected towards secondary side of valve terminating on the valve face, not reaching apices (Fig 14). Striae uniseriate, radiate throughout, 60-72 in 10 µm, forming a 73-83° angle with the raphe, composed of small rounded to transapically elongate (near raphe) areolae, 90-100 in 10 µm. Striae continue onto valve mantle and around apices (Fig 14). Exterior opening of fistula a short apically elongated slit, slightly eccentric in the central area between the central raphe ends on the
primary side of the valve, sometimes confluent with middle areola (Fig 14). *Internal view:* Straight raphe opens centrally within raised raphe-sternum ribs (Figs 13, 15-16). Prominent slightly expanded terminal nodules (Figs 13, 15). Central nodule apically elongated with rugose shallow central groove in line with raphe (Figs 13, 16). Virgae slightly raised on internal valve surface (Fig 16). Internal areola openings square to rounded (Fig 17). Internal fistula opening round (Figs 13, 16), located at the junction of the raphe-sternum rib and valve face, covered by domed structure when intact (Fig. 15).

**Holotype:** USNH D31023 slide deposited in the US National Herbarium (National Museum of Natural History, Smithsonian Institution in Washington, DC USA). The holotype is illustrated in Figures 1-2. Biological material preserved in 4% formaldehyde is also associated with holotype material.

**Isotype:** USNH D31022 slide [coordinates (37X, 116Y)] deposited in the US National Herbarium (National Museum of Natural History, Smithsonian Institution in Washington, DC USA).

**Reference strain:** Strain #BLCC-F88 (Berthold-Laughinghouse Culture Collection: Freshwater; University of Florida Algae Culture Collection), Davie, Florida USA

**Genbank Accessions from holotype material:** KY982278 (18S rRNA), MN251841 (rbcL)

**Type locality:** near S308 (Port Mayaca Lock), between Lake Okeechobee, FL and the C-44 Canal (St. Lucie Canal; 26.985639, -80.618171) with pH 8.2. Samples collected by N. R. Jungman during summer 2014.
**Etymology:** The epithet refers to this taxon's propensity for media with bicarbonate and pH 10 (Berthold et al. 2019; submitted).

**Distribution:** Known only from the type location in Lake Okeechobee (Florida, USA) here reported.

**Habitat:** Lives free-floating in water column and may often accumulate at surface because of high lipid levels. Cultured in media with salinity 1.4‰, 200mM sodium bicarbonate (200mM), and pH of 10 (300mM NaOH).
FIGURES 1–12. Phase contrast, LM, DIC, and fluorescent images of *Fistulifera alcalina*. Fig. 1. Phase contrast of holotype slide (NMNH#) material after oxidation. Fig. 2. Phase contrast of oxidized frustules of multiple single cells. Figs. 3-10. LM of cellular arrangement of varying cell numbers (Figs. 8-10 valves are in girdle view.). Fig. 11. Nile red fluorescence of lipid droplets showing red chlorophyll autofluorescence and yellow lipid fluorescence (excitation 450-490nm). Fig. 12. Autofluorescence of single chloroplast within cells (excitation 400-440nm). Scale bars = 10µm.
FIGURES 13–17. SEM micrographs of *Fistulifera alcalina* sp. nov. after hydrogen peroxide treatment. Fig. 13. Internal valve face with raised raphe sternum. Fig. 14. External valve face with slit-like fistula opening. Fig. 15. Internal valve face encircled with fimbriate margin and fistula covering. Fig. 16. Radial striae with rounded fistula and slightly bent raphe sternum. Fig. 17. Valve with square areolae. (Figs. 13-16) = 1µm; (Fig. 17) = 400nm.
3.2 Phylogenetic Analyses

In addition to morphological analyses, a more detailed taxonomic identification of *Fistulifera* sp. 154-3 was performed using both the 18S rRNA and the *rbcL* gene. The 18S rRNA gene shows that the genus *Fistulifera* is a well-supported (ML: 100%; BI: 1.0) monophyletic clade (box in Fig. 18) within the family Stauroneidaceae. *Fistulifera alcalina* (str. BLCC-F88) fell into a distinct subclade within *Fistulifera* sister to *Navicula* sp. JB12 (KF791556) and JB16 (KF791555) (ML: 60%; BI: 0.83).

Phylogenetic analysis of the *rbcL* gene sequence using both ML and BI also sustain *Fistulifera* as a well-supported genus (ML: 100%; BI: 1.0) monophyletic clade (box in Fig. 19). The phylogenetic analyses place *Fistulifera alcalina* sister to *F. solaris* and *F. saprophila* with high support (ML: 100%; BI: 1.0). *Fistulifera solaris* and *F. saprophila* are also separate and distinct species (ML: 61%; BI: 1.0).
FIGURE 18. Maximum likelihood and Bayesian inference of the phylogenetic relationship of the 18S gene sequence of *Fistulifera alcalina* and 45 diatom species including *Navicula lanceolata* (AY485484) and *Navicula reinhardii* (AM501976) as outgroup. Bootstrap support and posterior probabilities are cut off at 50 and 0.5, respectively at consensus nodes of both inferential analyses.
FIGURE 19. Maximum likelihood and Bayesian inference of the phylogenetic relationship of the rbcL gene sequence of Fistulifera alcalina and 22 diatom species including Navicula lanceolata C86 as outgroup (FJ002148). Bootstrap support and posterior probabilities are cut off at 50 and 0.5, respectively at consensus nodes of both inferential analyses.
### TABLE 2. Morphometric comparison of species within the *Fistulifera* genus. (-) indicates missing data. Live measurements are from live cells while remaining measurements are from oxidized material.

<table>
<thead>
<tr>
<th>Species</th>
<th><em>F. alcalina</em> sp. nov</th>
<th><em>F. iranensis</em></th>
<th><em>F. pelliculosa</em></th>
<th><em>F. saprophila</em></th>
<th><em>F. saprophila</em> BA55</th>
<th><em>F. saprophila</em> BA55</th>
<th><em>F. solaris</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Live Length</td>
<td>6.4 - 7.8 (7.2)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>8.2-9.3 (8.8)</td>
<td>8.2-9.3 (8.8)</td>
<td>6.4-7.1</td>
</tr>
<tr>
<td>Live Width</td>
<td>3.9 – 5.0 (4.4)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.1-4.1 (3.1)</td>
<td>2.1-4.1 (3.1)</td>
<td>3.2-4.4</td>
</tr>
<tr>
<td>Valve Length</td>
<td>4.2-6.4</td>
<td>9.7 - 11.2</td>
<td>8.9 – 12.5</td>
<td>4.5 – 7.6 (3.8)</td>
<td>4.5 - 7.6</td>
<td>4.9 - 7.6</td>
<td>5.2 - 6.5</td>
</tr>
<tr>
<td>Valve Width</td>
<td>2.3 - 3.0</td>
<td>3.1 - 4.0</td>
<td>4.0 – 6.2</td>
<td>2.0 - 4.0</td>
<td>2 - 4</td>
<td>1.4 - 3.2</td>
<td>1.61 - 2.23</td>
</tr>
<tr>
<td>Striae in 10µm</td>
<td>60 - 72</td>
<td>84</td>
<td>45-55</td>
<td>48-81</td>
<td>48 - 81</td>
<td>55-95</td>
<td>54 - 59</td>
</tr>
<tr>
<td>Striae angle 73 - 83°</td>
<td>90-100</td>
<td>-</td>
<td>45-50°</td>
<td>75-80°</td>
<td>75 - 80°</td>
<td>-</td>
<td>75 - 80°</td>
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<tr>
<td>Areolae in 10µm</td>
<td>105-123</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>80 - 110</td>
<td>80-120</td>
<td>80-90</td>
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<tr>
<td>Distance Between Areolae</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>111 - 125</td>
</tr>
<tr>
<td>Ecology</td>
<td>Brackish; Eutrophic</td>
<td>Euryhaline mesohalobes</td>
<td>low saprobity; beta mesoprobic; Oligotrophic</td>
<td>Highly Eutrophic</td>
<td>Eutrophic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Distribution/Habitat</td>
<td>Subtropical freshwater, brackish to alkaline</td>
<td>Saline spring; Iran</td>
<td>Cosmopolitan</td>
<td>Europe; Cosmopolitan</td>
<td>Europe; Cosmopolitan</td>
<td>Europe; Cosmopolitan</td>
<td></td>
</tr>
<tr>
<td>Media Salinity</td>
<td>1.4%</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.8%</td>
</tr>
<tr>
<td>Morphology</td>
<td>Solitary and chains</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Solitary</td>
</tr>
</tbody>
</table>
4. DISCUSSION

This study describes a new diatom contributing data on the ecology, morphology, and phylogeny of a small and often overlooked diatom taxon. Our work supports the monophyly of the genus *Fistulifera* (Figs. 18-19), which is composed of species that may be divided into freshwater, marine, and alkaline types, with much overlap in the tolerance for habitat water conductivity and saprobity (Table 2). This is the first record of a species of *Fistulifera* in the southeastern United States; species of the genus have been found in Connecticut (USA): *F. pelliculosa*; Ohio (USA), France, and the Czech Republic: *F. saprophila*; Iran: *F. iranensis*; and Japan: *F. solaris*.

*Fistulifera alcalina* was found in an artificially constructed and managed Lake Okeechobee outflow (Port Mayaca), thus extending this genus’ already cosmopolitan habitat. Lake Okeechobee is the “liquid heart” of South Florida, a central water supply system that feeds both the Everglades wetlands and urban agricultural demand. The lake undergoes a fluctuating pH that is attributed to the unique sub-tropical location of the lake and subsequent wet and dry seasons of South Florida (Havens et al. 2016). In South Florida, salinity and distance from the shoreline are drivers of specific diatom assemblages (Nodine & Gaiser 2014). Lake Okeechobee drainages are also abundant in pennate diatoms associated with disturbed boundary lines (Gaiser et al 2014); this is another example of a *Fistulifera* species that takes advantage of eutrophic or saprobic waters as *F. saprophila* (Szabo et al. 2005, Michelutti et al. 2007). Unlike the saprophytic species, *F. pelliculosa* is limited to oligotrophic waters, while *F. iranensis* and *F. solaris* are saline and marine species, respectively (Hustedt 1962, Matsumoto 2014). The
adaptation of this genus to varying physiochemical habitats, including organic pollution and increasing halo-alkaline conditions is apparent as *F. alcalina* can withstand salinity of 1.4‰ and pH 10 and since *F. saprophila* has been observed in brackish waters (Della Bella et al. 2007, Zgrundo et al. 2013).

*Fistulifera alcalina* is morphologically different than the other species of *Fistulifera* and the raphe sternum is the main difference. The raphe of *F. solaris* is much straighter than *F. alcalina; F. alcalina* is often observed with bent or curved raphe sternum and slits (Fig. 16). In *F. solaris*, the raphe sternum is connected to the central nodule through a shallow ditch (Matsumoto et al. 2014), whereas in *F. alcalina*, the raphe slits are joined together by a raised central nodule lacking a ditch (Fig. 15-16). Morphologically, *Fistulifera alcalina* differs from the other species of the genus in size (Table 2). Live cells of *F. alcalina* (L: 6.4-7.8; W: 3.9-5.0µm) are most similar to *F. solaris* in width (3.2-4.4 µm) and length (6.4-7.1 µm), but *Fistulifera alcalina* achieves larger dimensions. Though larger than *F. solaris*, live *F. alcalina* cells are much smaller in length than *F. saprophila* BA55 (L: 8.2-9.3; W: 2.1-4.1) but wider. When considering cell length after oxidation, dimensions indicate that *F. alcalina* is the shortest species in the genus *Fistulifera*, while the width of *F. alcalina* is similar to *F. saprophila* BA55 and *F. solaris*. Species of *Fistulifera* also differ in striae and areolae numbers: *F. alcalina* has 60-72 striae in 10 µm, while *F. solaris* has 54-59, *F. saprophila* 48-81, *F. iranensis* with 84, and *F. pelliculosa* with 45-55 striae in 10 µm. In terms of areolae, valves of *F. alcalina* have 90-100 areolae in 10 µm, while *F. solaris* has less with 80-90 in 10 µm and *F. saprophila* with a wider range of 80-120 in 10 µm. Lastly, the range of the distance
between areolae of *F. solaris* (111-125µm) falls into the range of *F. alcalina* (105-123µm), but *F. alcalina* generates smaller distances between areolae.

Phylogenetic inference of the 18S rRNA and *rbc*L genes maintained the monophyly of *Fitulifera*, as previously supported (Pniewski et al. 2010, Matsumoto et al. 2014) but also questioned by others (Rimet et al. 2011, Zgrundo et al. 2013). Both gene sequences supported the delimitation of *Fistulifera alcalina* sp. nov. (18S rRNA: ML 60%; BI 0.83; *rbc*L: ML: 100%; BI: 1.00). The 18S rRNA gene phylogeny revealed a distinct “alkaline” subclade in the genus that includes *F. alcalina* and two other strains, JB12 (KF791553) and JB16 (KF791555), which were isolated from high halo-alkaline soils (Qiao and Liu, GenBank). The 18S rRNA also resulted with species delimitation according to habitat water chemistry, comprised of freshwater species (BI: 0.82) including *F. saprophila* and *F. pelliculosa*, and the marine *F. solaris* (BI: 0.72), also reported by Matsumoto at al. (2014). Though we have extended our knowledge on the genus *Fistulifera*, this genus warrants further elucidation since very few long *rbc*L sequences are available. Further, species of the genus can be confused for other hyaline genera, such as *Eolimna* and *Mayamaea*, due to the small size and lack distinctive features in LM (Zgrungdo et al. 2013).
5. CONCLUSION

LM and SEM morphometrics along with 18S rRNA and rbcL molecular phylogenetic analyses support *Fistulifera alcalina* as a novel species. 18S rRNA phylogeny shows *Fistulifera alcalina* in a distinct clade of alkaline species. Our data support the monophyly of the genus *Fistulifera* that span the water electrochemical gradient: from freshwater, brackish, to saline, and now alkaline waters. In describing *Fistulifera alcalina*, we now have a more wholesome view into this genus and their distribution and habitat or preference.

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REFERENCES


III. CHAPTER 3

Bacterial co-culture effects on *Fistulifera alcalina* (Bacillariophyceae) oil productivity and composition
Bacterial co-culture effects on *Fistulifera alcalina* (Bacillariophyceae) oil productivity

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ABSTRACT

Diatoms are admirable for their growth rates and intracellular lipid generation. The characteristics and mechanisms that elicit high productivity in diatoms, especially alkaliphilic diatoms, are not well understood. A specific element of diatom productivity that warrants clarification is the presence of bacteria. Understanding the direct mechanisms of how bacteria affect diatom growth can provide insight into understanding the larger framework of diatom cultivation and multi-taxon consortia. To test the effects of bacteria on diatom growth and productivity, water column and diatom phycosphere-associated bacteria were isolated from Lake Okeechobee and co-cultured with *Fistulifera alcalina*. Results indicated the bacterium *Bacillus horikoshii* was able to stimulate the growth rate of *F. alcalina* while modulating the lipid content and isomers produced. These results demonstrate the effects of bacteria on diatom physiology with implications for the cultivation and success in the biotechnological field of diatoms.

**Key words:** fatty acids, lipids, alkaliphilic, growth rate, *Bacillus horikoshii*
1. INTRODUCTION

Diatoms are an exceptional biological model organism for growth and intracellular lipid generation (Stokes et al. 2020). The high growth rates and oil compounds produced by diatoms support aquatic food chains and are valued in human medical and nutraceutical industries (Malviya et al. 2016; Sprague et al. 2016). The characteristics and mechanisms that make diatoms prolific producers, specifically physiological drivers of growth rates and intracellular lipid biosynthesis, are not well understood. A specific element of diatom growth and oil productivity that warrants clarification is the presence of bacteria.

In nature, algal and bacterial interactions are widespread in marine and freshwater environments and extensively contribute to nutrient cycling (Sarmento and Gasol 2012). These complex microbial communities (MCs) influence large-scale global cycles of nitrogen, phosphorus, and other essential nutrients (Jørgensen 2006). The general qualities of algae and bacteria that enable their moderation of the environment include release of extracellular signaling molecules, enzymes, and byproducts while sequestering gases and nutrients from the environment. Understanding how algal and bacterial functional microbial characteristics such as signaling molecules intermingle in culture/cultivation or in the environment is essential in describing algal-bacterial relationships (Fuentes et al. 2016; Tisserand et al. 2020).

The shaping of function through metabolic states of MCs is recently considered a Metabolically Cohesive Consortia or MeCoCos (Pascual-Garcia et al. 2020). The MeCoCos that form in nature are described as stable, reproducible structures that are metabolically co-dependent and function together by both producing and using resources.
in a sustained manner (Pascual-Garcia et al. 2020). The MeCoCos members partake in specialized resources and roles which avoid competitive interactions and limit generalists. The concept of MeCoCos is especially important in the applied biotechnological field of algal cultivation. The role of beneficial, or “probiotic” bacteria is important for the success of algal growth (Lian et al. 2018). Through cellular attachment or involvement of extracellular cycling of specific compounds and elements including nutrients, dissolved organic carbon, vitamins, phytohormones, and secondary compounds, bacteria largely shape the physiology of algae (Lau et al. 2007; Tang et al. 2010; Teplitski and Rajamani 2011; Bagwell et al. 2016; Yang et al. 2016). Compounds involved in algal-bacterial interactions can be species-specific and dependent on the peripheral mucilage region of algae cells, or phycosphere (Bell and Mitchell 1972; Sapp et al. 2007) where extracellular polymeric substances (EPS) are often found. The species of bacteria found within algae EPS can also be specific to the interactions. The communication between algae and bacteria can be obligatory where diatom aggregation and biofilm formation, for example, are dependent on bacterial cell contact and exudates (Schnurr and Allen 2015; Windler et al. 2015; Yang et al. 2016). Though much is already known about algal-bacterial interactions (Zhang et al. 2020), the role of beneficial bacteria and their mechanisms remain largely unexplored in the context of alkaliphilic diatom growth and lipid generation (Marella et al. 2020).

The relationship between diatoms and bacteria is also an important consideration in biotechnological tool development since bacteria can stimulate lipid increases in algae (Kim et al. 2020; Marella et al. 2020). As bacterial presence is key to the growth and success of algae, microbial interactions with oleaginous algal species may be particularly
revealing for the physiological basis for diatom-bacterial interactions (Gonzales and Bashan 2000; Park et al. 2008; Amin et al. 2015). Bacteria have shown promising effects on algal growth rates and lipid production (Ward and Singh 2005; Natrah et al. 2014; Johansson et al. 2019; Berthold et al. 2019; Liu et al. 2020; Kim et al. 2020) and accelerating diatom growth rate is an ultimate goal in production schemes (Hannon et al. 2010). Most studies of microalgae associated MCs focused on salt/fresh water and wastewater, while only few studies have considered bacterial and algal relationships in an extremophilic or alkaliphilic environment.

Alkaliphilic algal and bacterial interactions are potentially a critical aspect for advances in algae biotechnology (Marella et al. 2002). The use of alkaliphilic diatoms in cultivation is an approach to limit the contamination that often occurs since high alkalinity reduces the persistence of contaminating microorganisms and stimulates the synthesis of oils (Gardner et al. 2011). There are few diatom strains proposed for large scale-cultivation and even fewer strains that are properly suited for high alkaline growth outside of wastewaters. To avoid the limitations brought by unwanted contamination in culture, extremophilic diatom species with the ability to persist where others cannot, and with a proclivity for high production in the presence of core bacteria are ultimately desired. To fulfill the necessity for robust alkaliphilic diatoms with improved growth rates in the presence of bacteria, more research focusing on bacteria associated with alkaliphilic diatoms, or diatom microbiomes, is necessary.

A recently described diatom species that has shown high growth rate in alkaline media is *Fistulifera alcalina* (Berthold et al. 2020). The diatom has demonstrated high growth rates with a moderate lipid productivity that can be increased in co-culture with
bacteria. Co-culture experiments are crucial in revealing the direct effects of bacteria on diatom physiology (Le Chevanton et al. 2013; Berthold et al. 2019; Liu et al. 2020). To test the effects of bacteria on *F. alcalina* and explore diatom and bacterial interactions in alkaline co-culture, the present research aimed to explore bacteria isolated from the type source of *F. alcalina*, Lake Okeechobee. Bacteria were isolated from the water column, as well as from other isolated diatoms and applied to *F. alcalina* to test the effects on the diatom’s growth and productivity. Understanding the direct mechanisms of how bacteria come together with diatoms to support their growth can provide insight into understanding the larger framework of diatom cultivation and multi-taxa consortia like MeCoCos. Exploring diatom microbiomes especially through isolation and phylogenetic interpretations also provides scientists with a repertoire of environment-specific bacteria for future consortia work, especially in alkaliphilic contexts.

2. MATERIALS AND METHODS

2.1 Isolation, maintenance, and microscopy

*Fistulifera alcalina* and other diatoms were isolated from several locations in the type habitat of Lake Okeechobee, FL, on 08/30/2017. Lake Okeechobee water samples were streaked on sterile solid (3% agar) source water and incubated until colonies formed. Individual colonies of diatoms were then transferred in modified BG11 (Blue-green algae media; Rippka et al. 1979) with the addition of NaSiO₃ (245 µM), Gambourg’s vitamin mix (Difco), and vitamin B-12. Diatom cultures were first identified to genus after oxidation with hot hydrogen peroxide (method citation) and light microscopy. Diatom isolates that appeared unique from each other were first identified on
the basis of morphology (Krammer and Bertalot 1991a,b; Round et al. 1990). Diatom cultures were maintained in 125ml flasks exposed to 50 µmol m² s⁻¹ fluorescent light without shaking. *Fistulifera alcalina* was maintained in modified Zarrouk’s media (MZ10) adjusted using NaOH (5.2 g L⁻¹; pH=10), NaSiO₃, Gambourg’s vitamin mix, and vitamin B12 with shaking at 120 rpm (Berthold et al. 2020).

Individual diatom isolates were used to isolate bacteria that were closely associated with the algal cell phycosphere. Xenic diatom cultures were first streaked onto nutrient agar with adjustment of pH 7, after isolation, the media was adjusted to pH 9 using 0.1M NaOH in order to match the favorable culture pH of *F. alcalina*. A total of 22 individual bacterial isolates from diatom cultures and the environment were procured and maintained on solid nutrient agar slants at room temperature (25°C) (Table 1). Of the 22 isolates, 14 bacterial isolates were derived from the phycosphere of isolated diatoms, with six isolates sourced from the initial Lake Okeechobee water samples, and two isolates were from the original culture media of *F. alcalina* (Table 1).

To visualize diatom lipid droplets, verify the presence of bacteria, and observe possible bacteria and diatom cell-to-cell interactions, algal and bacterial co-cultures were imaged using both light microscopy (LM) (Leica, DM5500 B) (LAS V4.12; Leica Microsystems, Switzerland) and epifluorescent microscopy (EFM) (Amscope, XYL-606). EFM was used to image diatom and bacterial co-cultures where Nile red and DAPI (4’,6-Diamidino-2-Phenylindole, Dihydrochloride) staining were simultaneously used for visualization of bacteria and diatom lipids.
2.2 Molecular and phylogenetic analyses

To identify the diatom source from which the bacteria were isolated, pure cultures of diatoms were subsequently identified taxonomically using both the 18S rRNA and the rbcL gene sequence phylogenies. To extract DNA, exponential phase diatoms biomass was transferred into an Eppendorf tube and washed twice with deionized water. Total algal genetic material was extracted using a Dneasy Plant Mini kit (Qiagen Inc, USA). The rbcL and 18S rRNA genes were selected for amplification using appropriate primers (Table 2).

Both rbcL and 18S rRNA gene sequences were amplified using a GoTaq PCR Core systems kit (Promega, USA) on a ProFlex thermocycler (Applied Biosystems, Life technologies). Methods for the rbcL followed Jones et al. (2005), with modifications. Briefly, 10 µL TBE buffer, 10 bovine serum albumin (BSA), 8 µL MgCl₂, 1 µL dNTPs, 0.25 µL GoTaq G2, 1µL template DNA, and 1 µL of each primer were pipetted into a PCR reaction tube. The PCR amplification involved an initial denaturation for 3 min at 94°C and final extension for 10 min at 72°C, and 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C. Methods for the 18S rRNA gene sequence can be found in Berthold et al. (2020). A QIAquick PCR Purification Kit was used to clean PCR products before visualization on a 0.8% agarose gel. Since Fistulifera species may be alldiploid, all 18S rRNA PCR products were subsequently cloned (Tanaka et al. 2015).

The amplified products of the 18S rRNA were cloned using TOP10 chemically competent cells prior to sequencing, following manufacture protocols (TOPO-TA, Invitrogen). Plasmids were extracted from clonal libraries using a PureLink Quick Plasmid Miniprep Kit (Invitrogen). Sequencing of the rbcL region and 18S rRNA
plasmid DNA was carried out using Sanger sequencing by Eurofins Genomics (Kentucky, USA) using BigDye Terminator v3.1 (Applied Biosystems). Sequences for both the 18S rRNA gene and rbcL gene regions are deposited in GenBank (National Center for Biotechnology Information, NCBI) (Table 2).

BLAST (Basic Local Alignment Search Tool, NCBI) was used to identify 18S rRNA and rbcL gene sequences similar to the isolates. The alignment of the 18S rRNA gene included up to 1892 sites, while that of the rbcL included 1426 sites. The best-fit nucleotide model was assessed using jModelTest through MEGA (v10.1.7) (Guindon and Gascuel 2003; Posada 2008; Stecher et al. 2020). A total of fifty-eight 18S rRNA sequences were aligned with the isolates using MUSCLE (SeaView v4.4) (Edgar 2004) with Navicula reinhardtii (AM501976) and Navicula lanceolata (AY485484) as outgroup. Similarly, twenty-eight rbcL diatom sequences were aligned with Navicula lanceolata (FJ002148) as outgroup. Phylogenies for both gene sequences were constructed using Bayesian inferencing (BI) and maximum likelihood (ML) with MrBayes (v3.3.6) and RaxML (7.2.7), respectively, through the CIPRES network (v.3.3) and MEGA, respectively (Guindon and Gascuel 2003; Miller et al. 2010). The ML analysis was carried out using the model using 1,000 bootstrap resampling replicates. The BI analysis was conducted using MrBayes 3.6 (Ronquist and Huelsenbeck 2003) with 1.0 x 10^6 generations, a 0.25 burn-in rate, and resampling every 100 generations.

The 16S rRNA gene sequence molecular phylogeny was used to identify the bacterial isolates. Bacterial isolates were identified by first streaking on solid nutrient agar and then transferring single colonies into sterile snap cap tubes containing 2 ml of nutrient broth (pH 9). Tubes were incubated on shaker at 200 rpm for 24 hrs at 30°C.
Cultures of individual bacteria were then transferred into sterile centrifuge tubes, and cell pellets washed once with biological grade water. Cell pellets were used for DNA extraction using a Dneasy UltraClean Microbial Kit (Qiagen). The 16S gene region was amplified using universal bacterial 16S ribosomal RNA primer 27F (5’- AGA GTT TGA TCC TGG CTC AG-3’) and 1392R (5’- GGT TAC CTT GTT ACG ACT T-3’) following PCR protocols of Taton et al. (2003). PCR products were sequenced as described above, and GenBank (NCBI) accession numbers for the 16S rRNA gene sequences are found in Table 1. BLAST was used as described above to locate similar bacterial 16S rRNA gene sequences (Table 1).

2.3 Screening co-cultures of bacteria with Fistulifera alcalina

To determine the potential effects of the bacterial isolates on the growth rate and lipid content of the diatom *F. alcalina*, 22 bacterial isolates were individually co-cultured with *F. alcalina* within 50 ml Erlenmeyer flasks. Prior to co-culturing, individual bacterial isolates were inoculated into 2 ml of sterile nutrient broth (pH 9) within snap cap tubes and incubated on shaker at 200 rpm at 25°C for 48 hours. After incubation, bacterial cultures were centrifuged, and the bacterial pellet was washed three times with sterile MZ10 and diluted to an optical density of 600nm (OD$_{600}$) of (0.02). An exponential phase of *F. alcalina* was aliquoted into 50-mL sterile Erlenmeyer flasks containing MZ10 until OD$_{440nm}$ of 0.1 was reached, followed by 0.5 mL aliquot of individual bacterial suspensions using triplicates. The co-cultures were then placed on a shaker at 120 rpm under 75 μmol photons m$^{-2}$ s$^{-1}$ at 25°C for nine days.
The growth rate of *F. alcalina* was monitored using OD$_{440}$ and filtering 1ml of each treatment onto a pre-weighted glass microfiber filter (Whatman/GF-C 1.2 µm) dried to constant weight. Lipid content of each treatment was determined using the Nile Red method of lipid staining described in Berthold et al. (2019) (Chen et al. 2009). Briefly, 1ml of homogenized algal culture was treated with DMSO (20%) and then stained with Nile red (10 µg mL$^{-1}$) and the absorbance and fluorescence read using a plate reader (Biotek; Synergy HTX) at excitation and emission wavelengths of 530 nm and 575, using neutral lipid standards. A triolein lipid standard cubed was used to translate the fluorescence present in 100 µL cell suspension into lipid content (% of dry biomass). Lipid productivity (mg L$^{-1}$ day$^{-1}$) was calculated based on biomass productivity (g L$^{-1}$ day$^{-1}$) and lipid content (% dry biomass).

### 2.4 Bacterial cell number effects on *Fistulifera alcalina* productivity

Prior to co-culture and gravimetric determinations, the effect of bacterial cell number on the growth and lipid productivity of *F. alcalina* was first evaluated to inoculate future experiments in favorable cell concentrations. A 1-L batch culture of *Bacillus horikoshii* B13 was cultured in MZ10 with ¼ nutrient broth for 24 hours at 120 rpm. Cells were harvested by centrifugation and aseptically washed twice with MZ10. Exponential cultures of *F. alcalina* were inoculated in 75 ml of MZ10 at a concentration of 10$^5$ cells/ml. Bacteria cells were inoculated at four different bacteria: algae ratios using triplicates including 1:100, 1:1, 100:1, and 10,000:1 (corresponding to 10$^3$, 10$^5$, 10$^7$, 10$^9$ bacteria cells/ml, respectively).
Growth and lipid productivity of microalgae and bacterial co-cultures were monitored every other day for 9 days and evaluated gravimetrically and using Nile Red, respectively (as described in section 3.3) with the addition of cell enumeration using a Bürker-Türk counting chamber for algal cells. Bacteria cell concentration was enumerated using modified protocols of direct epifluorescent filter technique (DEFT) (Chen et al. 2001) and CFU (colony forming units) counting using nutrient agar (pH 10). SYBR gold (10,000X in DMSO; Life technologies) was first diluted to a 100X working stock using TE buffer (stored at -20°C). For bacterial cell staining, 500 µL of each sample was transferred into a microcentrifuge tube and fixed with formaldehyde (final concentration 2%) for 1 hr. Samples were diluted using PBS buffer and subsequently stained with SYBR gold (2X) for 15 min. A 100 µL aliquot of the stained sample was then placed on top of a polycarbonate filter into a water droplet and gently (100 mm Hg) vacuumed completely. Polycarbonate filters were air dried for 1 min and then affixed to glass slides smeared with glycerol and bacteria were enumerated using EFM (450-490nm).

2.5 Effects of bacterial filtrate on Fistulifera alcalina in co-culture

The most influential bacterium (Bacillus horikoshii B13) in stimulating the growth and/or lipid productivity of F. alcalina from section 3.3 was chosen to upscale in quantity for gravimetric analyses of lipid content and profile. To observe the potential effects this bacterial isolate on F. alcalina, an exponential culture of the diatom was inoculated in triplicates with initial OD$_{600}$ 0.1 into 6- 1.5 L flasks containing 1200mL of MZ10. Co-cultures with mixed diatom and bacterial cells were then inoculated at a
bacterial to algal cell ratio of 100:1 (10⁷ and 10⁵ bacterial to algal cells/ml respectively). Flasks were shaken 75 rpm under 12:12 light dark lighting of 75 mol photons m⁻² s⁻¹ at 25 °C. Biomass and lipid productivities were assessed as previously described (Section 3.3) with monitoring every odd day for 9 days. Cultures were harvested on day 9 by centrifugation at 8000g (Beckman Coulter, Avanti J-E) and biomass paste was then transferred into pre-weighted 50 ml centrifuge tubes, frozen and subsequently lyophilized for lipid analysis.

### 2.7 Statistical analyses

To determine significant differences between treatments including lipid content, biomass and lipid productivity for the screening of 22 bacterial strains in co-culture with *F. alcalina* (Section 2.3), a repeated measure ANOVA was used. To determine significant differences between treatments including lipid content, biomass and lipid productivity, diatom and bacterial cells counts to determine the effects of *B. horikoshii* cell concentration on with *F. alcalina* in co-culture (Section 2.4), a repeated measure ANOVA was also used. A repeated measure ANOVA was similarly applied to determine significant different among treatments in section 2.5 and 2.6. Differences between treatment means for each variable, day, and section were compared using Kruskal-Wallace multiple comparisons with significance at p<0.05. All statistical analyses were performed using R (R team 2017).
3. RESULTS

3.1 Molecular and phylogenetic characterizations

A total of seven diatom strains were isolated in this work to obtain diatom phycosphere-related bacteria (Table 1). Amplification of both the 18S rRNA and the \( rbcL \) gene sequences resulted with sequences ranging up to 1892 and 1426 bp in length, respectively. BLAST analysis of one diatom strain revealed a 100% match to \textit{Nitzchia palea} (BLCC-F142), while the remaining four isolates all demonstrated 99.8% or higher similarities to the genus \textit{Fistulifera}. All of the \textit{Fistulifera} isolates demonstrated high concentrations of lipids within the cells compared to the known oleaginous \textit{F. alcalina} (Figure 1).

<table>
<thead>
<tr>
<th>Diatom CC ID</th>
<th>Taxa</th>
<th>18S</th>
<th>( rbcL )</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLCC-F88</td>
<td>\textit{Fistulifera alcalina}</td>
<td>MT742237</td>
<td>MN251841</td>
</tr>
<tr>
<td>BLCC-F141</td>
<td>\textit{Fistulifera sp. nov}</td>
<td>tbd</td>
<td>tbd</td>
</tr>
<tr>
<td>BLCC-F142</td>
<td>\textit{Nitzchia palea}</td>
<td>tbd</td>
<td>-</td>
</tr>
<tr>
<td>BLCC-F143</td>
<td>\textit{Fistulifera solaris}</td>
<td>tbd</td>
<td>tbd</td>
</tr>
<tr>
<td>BLCC-F144</td>
<td>\textit{Fistulifera sp. nov}</td>
<td>tbd</td>
<td>tbd</td>
</tr>
<tr>
<td>BLCC-F145</td>
<td>\textit{Fistulifera solaris}</td>
<td>tbd</td>
<td>tbd</td>
</tr>
<tr>
<td>BLCC-F146</td>
<td>\textit{Neidium sp.}</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>BLCC-F147</td>
<td>\textit{Cyclotella sp.}</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

(-) denotes not determined.
Figure 1 Brightfield (left) and fluorescent (right) microscope images of *Fistulifera* diatom species isolated from Lake Okeechobee showing red autofluorescence of chlorophyll and yellow fluorescence of lipid droplets. Specimens include *Fistulifera alcalina* sp. BLCC-F88 (A) *Fistulifera* sp. BLCC-141 (B), *Fistulifera* sp. BLCC-143 (C), *Fistulifera* sp. BLCC-F144 (D), *Fistulifera* sp. BLCC-F145 I. [Nitzschia palea BLCC-F142, Neidium sp. BLCC-F146, and Cyclotella sp. BLCC-F147 are not shown]. Scale bars represent 40 µm.
Molecular phylogenetic analysis of the 18S rRNA gene sequence demonstrated a well-supported monophyletic clade for the genus *Fistulifera* (ML: 100%; BI: 1.0) within the family Stauroneidaceae. (Fig. 2). Within this monophyletic clade, two isolates formed a well-supported clade with *F. solaris* (BLCC-F143, BLCC-F145) (ML: 58%; BI: 0.62). *Fistulifera alcalina* retained monophyly (ML: 100%; BI: 1.0). The remaining two isolates (including two clones from one strain) together formed a well-supported clade with high support (BLCC-F141, BLCC-F144) (ML: 84%; BI: 1.0).
Figure 2 BI phylogenetic tree of the 18S rRNA gene sequence of isolated *Fistulifera* strains using *Navicula lanceolata* and *N. reinhardtii* as outgroup. Both posterior probabilities and bootstrapping at consensus nodes are cut off at 50%/0.5.
Similarly, the rbcL gene sequence phylogeny demonstrated a monophyletic *Fistulifera* clade with high support (Fig. 3; ML: 100%; BI: 1.0). *Fistulifera alcalina* delimited from the remaining species with high support (ML: 100%; BI: 1.0). The purported *F. solaris* species formed a well-supported sub-clade containing *F. solaris*, and *F. solaris* str. BLCC-F143 and BLCC-F145 (ML: 99%; BI: 1.0). *Fistulifera* strains BLCC-F141 and BLCC-F144 also formed a well-supported clade (ML: 60%; BI: 1.0).

**Figure 3** BI phylogenetic tree of the rbcL gene sequence of isolated *Fistulifera* strains using *Navicula lanceolata* C86 as outgroup. Both posterior probabilities and bootstrapping at consensus nodes are cut off at 50%/0.5.
Amplification of the bacterial 16S rRNA gene resulted with gene sequences ranging from 1351-1434 bps in length. BLAST analyses of each bacterial isolate demonstrated genetic similarities of 99.2 or greater for each isolate corresponding to bacteria taxa listed in Table 2. The most notable bacterial strain in stimulating *F. alcalina*, demonstrated a 16S rRNA gene phylogeny that suggests *Bacillus horikoshii* (Fig. 4) with high support, though *B. horikoshii* appears polyphyletic.
Table 2. Bacterial strain information including bacterial ID number for this project, BLAST ID based on 99.7% identity cutoff, culture collection number of bacterial strains, bacterial source, and the source strain or location ID of the bacterium.

<table>
<thead>
<tr>
<th>Bacteria ID#</th>
<th>BLAST ID (&gt;99.7)</th>
<th>CC#</th>
<th>Bacterial Source</th>
<th>Source Strain ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td><em>Pseudomonas alcaligenes</em></td>
<td>BA</td>
<td></td>
<td><em>Fistulifera BLCC-F141</em></td>
</tr>
<tr>
<td>B2</td>
<td><em>Pseudomonas peli</em></td>
<td>GA</td>
<td></td>
<td><em>Nitzchia palea BLCC-F142</em></td>
</tr>
<tr>
<td>B3</td>
<td><em>Hydrogenophaga sp.</em></td>
<td>GB</td>
<td><em>Nitzchia palea</em></td>
<td><em>BLCC-F142</em></td>
</tr>
<tr>
<td>B4</td>
<td><em>Porphyrobacter sp.</em></td>
<td>GC</td>
<td></td>
<td><em>Nitzchia palea BLCC-F142</em></td>
</tr>
<tr>
<td>B5</td>
<td><em>Pseudomonas alcaligenes</em></td>
<td>HA</td>
<td></td>
<td><em>Fistulifera solaris BLCC-F143</em></td>
</tr>
<tr>
<td>B6</td>
<td><em>Kinneteria asaccharophila</em></td>
<td>QA</td>
<td></td>
<td><em>Fistulifera BLCC-F144</em></td>
</tr>
<tr>
<td>B7</td>
<td><em>Bacillus indicus</em></td>
<td>QB</td>
<td></td>
<td><em>Fistulifera BLCC-F144</em></td>
</tr>
<tr>
<td>B8</td>
<td><em>Pseudomonas alcaligenes</em></td>
<td>QC</td>
<td></td>
<td><em>Fistulifera BLCC-F144</em></td>
</tr>
<tr>
<td>B9</td>
<td><em>Pseudomonas alcaligenes</em></td>
<td>NB</td>
<td></td>
<td><em>Fistulifera BLCC-F145</em></td>
</tr>
<tr>
<td>B10</td>
<td><em>Pseudomonas otitidis</em></td>
<td>SB</td>
<td></td>
<td><em>Cyclotella sp. BLCC-F147</em></td>
</tr>
<tr>
<td>B11</td>
<td><em>Bacillus horikoshii</em></td>
<td>F88A</td>
<td></td>
<td><em>Fistulifera alcalina BLCC-F88</em></td>
</tr>
<tr>
<td>B12</td>
<td><em>Bacillus pseudofirmus</em></td>
<td>F88B</td>
<td></td>
<td><em>Fistulifera alcalina BLCC-F88</em></td>
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<tr>
<td>B13</td>
<td><em>Bacillus horikoshii</em></td>
<td>F88C</td>
<td></td>
<td><em>Fistulifera alcalina F88</em></td>
</tr>
<tr>
<td>B31</td>
<td><em>Hydrogenophaga sp.</em></td>
<td>RA</td>
<td></td>
<td><em>Neidium sp. BLCC-F146</em></td>
</tr>
<tr>
<td>B17</td>
<td><em>Xanthomonas sp.</em></td>
<td>F88D</td>
<td></td>
<td><em>Fistulifera alcalina BLCC-F88</em></td>
</tr>
<tr>
<td>B18</td>
<td><em>Pseudomonas mendocina</em></td>
<td>F88E</td>
<td>Bioreactor</td>
<td><em>Fistulifera alcalina BLCC-F88</em></td>
</tr>
<tr>
<td>B19</td>
<td><em>Aeromonas veronii</em></td>
<td>LO17-1A</td>
<td></td>
<td>Lake Okeechobee</td>
</tr>
<tr>
<td>B20</td>
<td><em>Aeromonas veronii</em></td>
<td>LO17-1B</td>
<td></td>
<td>Lake Okeechobee</td>
</tr>
<tr>
<td>B21</td>
<td><em>Acinetobacter sp.</em></td>
<td>LO17-2B</td>
<td>Water</td>
<td>Lake Okeechobee</td>
</tr>
<tr>
<td>B22</td>
<td><em>Exiguobacterium sp.</em></td>
<td>LO17-2C</td>
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</tr>
<tr>
<td>B23</td>
<td><em>Bacillus cereus</em></td>
<td>LO17-2F</td>
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</tr>
<tr>
<td>B26</td>
<td><em>Pseudomonas montellii</em></td>
<td>LOA-2</td>
<td></td>
<td>Lake Okeechobee</td>
</tr>
</tbody>
</table>
3.2 Screening of bacterial cocultures with F. alcalina

A total of 22 bacterial isolates, representing 16 different bacterial species, were used to screen the productivity of F. alcalina in co-culture with bacteria (Table 2). Overall, there were significant differences between the bacterial strains screened in lipid content, biomass productivity, and lipid productivity (P=0.00137, <0.001, <0.001, respectively) over time, but was not significant between all variables on an individual day basis.
Altogether, the bacterial strains either resulted in stimulation in diatom lipid content or biomass productivity, but not both simultaneously (Supplemental Table 1). Mean difference among co-culture treatments in comparison to diatom monoculture ranged from -26.68 to 19.53% for lipid productivity, -20.8 to 19.5% for biomass productivity, and -23.8 to 34.4% for lipid content. The lowest lipid productivity occurred in Pseudomonas alcaligenes str. BLCC-B1 co-cultures. Such a low lipid productivity in the co-culture resulted in light of both reduced biomass productivity and lipid contents in comparison to axenic cultures (-4.58% and -26.68%, respectively). The highest average lipid productivity occurred in co-cultures of Bacillus horikoshii str. BLCC-B13, with a 19.5% increase over the control. The increase in co-cultures with B. horikoshii str. BLCC-B13 was a result of stable lipid content (-0.49% difference), and a biomass productivity increase of 15.8%. The remaining bacterial co-cultures resulted in either higher or lower lipid productivities based on relatively larger losses of either lipid content or biomass productivity. Since Bacillus horikoshii str. B13 demonstrated improved lipid productivities within Fistulifera alcalina, based on a large increase in biomass productivity with little effect on lipid content, B. horikoshii was selected for subsequent experiments.
3.3 Effects of Bacillus horikoshii str. BLCC-B13 cell concentration on *F. alcalina* productivity

To properly evaluate the presence of bacterial cells and the possible effects on the biomass and lipid productivity of *F. alcalina*, different initial bacterial-to-algal cell ratios were evaluated 1:100 1:1, 100:1, and 10,000:1 corresponding to $10^3$, $10^5$, $10^7$, $10^9$ bacterial cells/ml, respectively. Between the axenic and co-cultures, there were no significant differences in lipid content ($P=0.675$) and lipid productivity ($P=0.0523$) over nine days of cultivation. All varying bacterial-to-algal cell ratio treatments indicated an increase in lipid productivity compared to axenic cultures (Table 3). The most productive cell ratio treatment was 100:1 bacteria-to-diatom with a 21% increase in productivity compared to axenic cultures. The remaining treatments resulted in an average increase of 14%, 12%, and 8% for 10,000:1, 1:1, and 1:100 cell ratios respectively. There was a significant difference in biomass productivity ($P=<0.001$) among treatments over time, but never among treatments pertaining to the same day.
Table 3. Average lipid content, biomass, and lipid productivity of *Fistulifera alcalina* in co-culture with increasing cells of *Bacillus horikoshii* str. BLCC-B13 over 9 days of culture. Data shown is mean of three replicates.

<table>
<thead>
<tr>
<th>Bacterial concentration (cells mL⁻¹)</th>
<th>Lipid content (%)</th>
<th>Biomass productivity g L⁻¹d⁻¹</th>
<th>Lipid productivity mg L⁻¹d⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axenic</td>
<td>39.7</td>
<td>0.24</td>
<td>87.4</td>
</tr>
<tr>
<td>10³</td>
<td>42.6</td>
<td>0.24</td>
<td>94.5</td>
</tr>
<tr>
<td>10⁵</td>
<td>40.1</td>
<td>0.25</td>
<td>97.4</td>
</tr>
<tr>
<td>10⁷</td>
<td>39.1</td>
<td>0.33</td>
<td>106.0</td>
</tr>
<tr>
<td>10⁹</td>
<td>39.1</td>
<td>0.29</td>
<td>99.6</td>
</tr>
</tbody>
</table>

Besides cell lipid content and biomass and lipid productivity, the cell concentrations of both diatom (Fig. 5) and bacterial (Fig. 6) cells changed throughout the nine-day cultivation. Concentration of diatom cells over the nine-day cultivation in varying bacterial cell concentrations were significantly (P=<0.001) different over time but not among the treatments on an individual day basis. The average bacterial specific growth rates over nine days for *Fistulifera alcalina* in axenic cultures and in increasing bacterial cell concentrations of 10³, 10⁵, 10⁷, 10⁹ was 0.35, 0.35, 0.34, 0.37, 0.35 cells mL⁻¹ d⁻¹, respectively.
Over the nine-day cultivation there were no significant difference in bacteria cell (P=0.889) concentration among co-cultures of *F. alcalina* with increasing inoculum of *B. horikoshii*. The average bacterial specific growth rates over nine days for increasing *B. horikoshii* cell concentrations $10^3$, $10^5$, $10^7$, $10^9$ was -0.15, 0.32, 0.04, 0.14 cells mL$^{-1}$ d$^{-1}$, respectively.
Figure 6 Bacillus horikoshii str. BLCC-B13 cell concentrations (cells mL⁻¹) in co-culture with Fistulifera alcalina inoculated with increasing initial cell concentrations (10³, 10⁵, 10⁷, 10⁹) of Bacillus horikoshii BLCC-B13 over nine days of culture.

3.4 Effects of Bacillus horikoshii str. B13 on Fistulifera alcalina

The supernatant of Bacillus horikoshii was tested on Fistulifera alcalina in larger culture vessels (2L flask) and resulted in the death of F. alcalina after the first day after inoculation and therefore excluded. Conversely, co-cultures of F. alcalina and B. horikoshii in larger culture vessels persisted but revealed no significant differences between the axenic and the xenic cultures in lipid content, cell concentrations, biomass, and lipid productivity (data not shown).
The total DW of the treatments after nine days of cultivation was 0.78 and 0.75 g L⁻¹ for the axenic and co-cultures, respectively (Fig. 7). The average lipid content over nine days of cultivation for axenic and co-cultures were 25.5 and 27.8%, respectively. The average biomass and lipid productivity for axenic and co-cultures were 0.27 and 0.24 g L⁻¹d⁻¹ respectively for biomass, and 61.6 and 61.5 mg L⁻¹d⁻¹ respectively for lipids.

3.5 Lipid profile of Fistulifera alcalina in co-culture with Bacillus horikoshii str. B13

Figure 8 Lipid profile of Fistulifera alcalina in axenic and co-culture with Bacillus horikoshii str. BLCC-B13. Results are based on lyophilized biomass harvested after nine days of cultivation.
4. DISCUSSION

4.1 Algal and microbial phylogeny and diversity

Seven strains of naturally occurring diatoms from the type environment of *F. alcalina* were isolated and cultured in this work to procure bacteria in order to screen against *F. alcalina*. The 18S and rbcL phylogenetic relationships, revealed high alpha diversity for the genus *Fistulifera* (Fig. 1 and 2) from Lake Okeechobee. The 18S gene sequence also indicated that some *Fistulifera* species contain several copies of the 18S gene and are aneuploids, as seen in the allodiploid *Fistulifera* and other diatoms (Tanaka et al. 2015). These results suggest that Lake Okeechobee waters provide the necessary chemistry to sustain a diverse array of species in the *Fistulifera* genus (*F. solaris*, *alcalina*, *saprophila*, and unknown). A result that is not surprising as the marine *F. solaris* indicated tolerance for low salinity (Satoh et al. 2013), and *F. saprophila*’s very epithet stands for the ability to withstand waters with high organic pollution, both characteristics of which are present in Lake Okeechobee. These results also bring into question the endemism of diatom strains and the scientific endeavor to delineate species based on water chemistry or habit or habitat type. Conversely, cosmopolitan raphid diatoms such as *Pseudo-nitzschia pungens* have demonstrated interbreeding of different clades in “hybrid zones” away from their well-known environments (Kim et al. 2020). Perhaps the *Fistulifera* genus takes advantage of breeding zones such as Lake Okeechobee as strategy to maintain stable genetic diversity within the genus. This work showed that the genus *Fistulifera* is cosmopolitan and made up of either benthic/planktonic, single-celled/colony-forming, and freshwater/brackish/saline species
that can all co-occur in one habitat. These tiny diatoms are able to accumulate high concentrations of lipid within their cells (Figure 1), and whether their lipid productivities and physiological constraints, such as media pH, match that of *F. alcalina* is impetus for future work.

The 16S rRNA gene sequence data showed that most of the bacterial species associated with the diatoms isolated in this work are either alkaliphilic, alkalitolerant, or halotolerant, a result that could be indicative of the source environment or the media used for isolation. Furthermore, the 16S gene phylogeny indicated that *Bacillus horikoshii* B13, was the most effective increasing the lipid productivity of *F. alcalina* through the increase of specific growth, although the 16S phylogeny indicates polyphyly among *Bacillus horikoshii* strains (Fig. 3).

### 4.2 Screening of 22 bacterial strains in co-culture with *F. alcalina*

Screening experiments using *F. alcalina* and 22 bacteria strains isolated from Lake Okeechobee water column and diatom phycospheres demonstrated that no single bacterial strain was effective at stimulating both biomass and lipid productivity. The results from this screening experiment is not surprising, as it was previously observed that very few bacteria can elicit simultaneous biomass and lipid generation in algae (Berthold et al. 2019). These results suggest that it may be necessary to target bacteria that can either stimulate biomass or lipid content of this diatom, or that targeting core consortia might be a better approach to achieve more reproducible results (Mönnich et al. 2020; Pascual-García et al. 2020). In terms of increasing productivity, *Bacillus horikoshii*
str. BLCC-B13 was the most efficient bacterium at increasing the productivity of *F. alcalina* by at least 20%. To further investigate the potential effects of this bacterium on the diatom, increasing cell ratios were subsequently co-cultured with *F. alcalina*.

### 4.3 Effects of *B. horikoshii* on *F. alcalina* productivity in co-culture

To evaluate the presence of bacterial cells and the possible effects on the biomass and lipid productivity of *F. alcalina*, different initial bacterial-to-algal cell ratios were evaluated 1:100 1:1, 100:1, and 10,000:1 corresponding to $10^3$, $10^5$, $10^7$, $10^9$ bacterial cells/ml, respectively. In terms of lipid content (% DW), axenic and co-cultures with increasing bacterial cells did not differ significantly, a result that was also observed in the screening of *F. alcalina* (section 3.2) where the overall difference between *B. horikoshii* co-cultures and the axenic cultures was 0.41%. In terms of biomass productivity, bacteria-to-algae cell ratios of 100:1 and 10,000:1 on average were able to increase the biomass productivity by 38 and 21% respectively. The increase in biomass productivity was also observed in section 3.2, where *B. horikoshii* increased the biomass productivity by 16% over *F. alcalina* axenic cultures. In terms of lipid productivity, cell ratios of 100:1 and 10,000:1 were the most effective at increasing the lipid productivity within *F. alcalina* by 21 and 14%, respectively, which mirrors the productivity increase of 20% observed in section 3.2 between co-cultures of *B. horikoshii* and *F. alcalina*.

In terms of cell numbers, co-cultures of *B. horikoshii* and *F. alcalina* demonstrated increased diatom specific growth rates in co-cultures with $10^7$ bacterial cells, corresponding to cells ratios of 100:1, from 0.35 to 0.37 cells mL$^{-1}$ d$^{-1}$. The
remaining cell ratios (1:100, 1:1, and 10,000:1) all demonstrated similar specific growth rates as the axenic cultures. Conversely, the specific growth rate of the bacterium \emph{B. horikoshii} differed among the increasing bacterial-to-algal cell ratios. On average, the specific growth rate of the bacterium was negative for the 1:100 cell ratio which signifies a decline in bacterial cell numbers through time. The cell ratio of 1:100 first started with low bacterial cell numbers and rose quickly between day 3 and 5, with a large decline thereafter. The cultures with cell ratio of 1:1 had the highest growth rate among the treatments but began to decline after 7 days of cultivation. The cultures with cell ratio of 10,000:1 cell ratio also had a positive specific growth rate, but greatly declined after initial inoculation from $10^9$ bacterial cells to $10^7$ and then $10^6$ on the third day. The cultures with cell ratio of 100:1, without sharp increases or declines, demonstrated a relatively stable specific growth rate over the cultivation period. These results demonstrate that initial inoculum cell concentration of \emph{B. horikoshii} affects the specific growth rate of \emph{F. alcalina}. From these results, inoculum with too low (1:100) and too high (10,000:1) bacterial-to-algal cell ratios can result in irregular cell growth in both diatoms and bacteria, while 100:1 cell ratios can result in improved diatom growth with stable bacterial presence. Altogether these results maintain that \emph{B. horikoshii} does not affect lipid content in \emph{F. alcalina} but rather can stimulate biomass production which is responsible for lipid productivity improvements observed.

The stimulation in diatom growth can be a result of many factors including the contribution of dissolved inorganic carbon (DIC) or involvement of extracellular enzymes. DIC could have played a role in providing the diatom with the photosynthetic boost needed when areal carbon dissolution into the media was not enough. As both
organisms in the co-cultures used are alkaliphilic, the production of extracellular alkaline proteases by the bacterium can also potentially be a useful aspect for the diatom (Joo et al. 2002). Enzymes are crucial industrial commodities, and alkaline proteases are central as they function in alkaline environments (Sharma et al. 2019). The bacterial alkaline proteases are a potential tool for the diatom to access nutrients that would otherwise be unavailable. Bacterial proteases could also be used by the diatom to supplement its very own production of alkaline proteases. Proteases are helpful in alkaline environments with organic matter, where their breakdown provide peptides and amino acids (Padan et al. 2005). The breakdown of proteins can be especially helpful in an environment such as the eutrophic Lake Okeechobee, with fluctuating pH and high organic matter content. This data suggests the relationship between *F. alcalina* and *B. horikoshii* is one that possibly does not involve stimulatory secondary compounds, but rather a physical transformation of necessary compounds or elements, or removal of waste (Danger et al. 2007).

Another physiological feature often overlooked when considering algal and bacterial interactions is pH. Soil pH is a crucial aspect of soil function including enzyme activity, nutrient availability, and consequent bacterial activity (Neina 2019), which should also extend to include aquatic environments. *Fistulifera alcalina* and *B. horikoshii* were cultured in media with high pH (10.3) and could possible reflect that of soils with high alkaline pH that allow for better nutrient availability. In this light, nutrients are freely accessible to both organisms and *F. alcalina* does not require close bacterial interactions, but rather scavenges for required nutrients. It is understood that the trophic level of the water and the consequent nutrient level can greatly govern the association
between bacteria and algae (Unnithan et al. 2014). In this light, more research is needed to reveal the dynamics of algal/bacterial interactions including nutrient availability in context of high alkaline media.

Cell ratios at inoculation are an important factor in the growth of algae in context of the cultivation function (Su et al. 2012) but algal production can also be limited by several physiological environmental variables including operational factors like vessel size and surface-to-air volume ratio (Park et al. 2011; Arandia-gorostidi et al. 2020). From the screening experiment to the cell ratio experiment, different results were obtained from the scaled-up growth of the co-cultures, results also previously observed (Berthold 2019). Since the cell concentration of both bacteria and algae were identical in all experiments, the disparity in biomass and lipid productivity in the larger scale cultivation most likely came from differences in cultivation vessel size that altered the surface-to-volume ratio of the media. Surface-to-volume ratio can greatly influence the productivity of algae (Venancio et al. 2020). To achieve a more comprehensive understanding of algal/bacterial relationships, it is essential to scale laboratory co-culture or consortia experiments to pilot-scale studies (Pham et al. 2019). Future work needs to take into consideration the chemical properties of the media and its effect on nutrients and gas availably, the varying scales of experiment and the confounding effects of cultivation parameters like surface-to-air volume ratios.

To further test the effects of B. horikoshii on the physiology of F. alcalina, the diatom was inoculated in bacterial supernatant to demonstrate potential effects of extracellular mechanisms (Berthold et al. 2019; Park et al. 2008). In the presence of B.
horikoshii filtrate F. alcalina died suggesting toxigenic properties of this bacterium previously shown to produce tetrodotoxins (Lu and Yi 2009). These results indicate that culturing parameters are going to largely affect the association between diatoms and bacteria in co-culture, and that individual examination of co-cultures can be crucial in unveiling the nature of bacterial and algal relationships and potential obstacles of scaling-up. More work is also needed to distinguish the physiological effects of alkaline media on algal and bacterial physiology and consequent interactions.

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IV. CHAPTER 4

Comparative mitochondrial genomics of species of the diatom genus *Fistulifera*
Comparative mitochondrial genomics of species of the diatom genus *Fistulifera*

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**ABSTRACT**

Diatoms are stramenopile algae with high growth rate and lipid generating properties. The mitochondrion is involved in the efficacy of diatom respiration and lipid synthesis and especially important in understanding diatom physiology and evolution. Exploring the mitochondrial genomes (mitogenomes) of diatoms and applying comparative genomics provide new insights into the molecular framework of diatoms. A lack of comparative genomic studies exists in the context of comparing oleaginous or extremophilic diatoms. To provide data on a novel alkaliophilic diatom, *Fistulifera alcalina*, the mitogenome was sequenced and compared to its sister species, *F. solaris*. Comparative analyses of *Fistulifera* mitogenomes revealed collinear synteny, with 83% similarity, and nearly a 2 Kb reduction in the noncoding region of *F. alcalina*. Pairwise alignment of *Fistulifera* mitogenomes also revealed a high level of polymorphic sites across the genome, especially in the *nad2* gene. Results suggest that the alkaliophilic diatom *F. alcalina* may have a streamlined mitochondrial genome in comparison to the marine species.

**Key words:** whole genome, alkaliophilic, mitochondria, *nad2*, SNPs, *F. alcalina*,
1. INTRODUCTION

Diatoms are exclusive stramenopile algae with high specific growth and lipid generating properties that make them contenders in the cultivation of algae (Hildebrand 2012). There are many diatom strains proposed for large scale-cultivation, but few strains are properly suited because of their incapacity for simultaneous fast growth and high lipid content (Griffiths and Harrison 2009). To advance diatom strain performance in culture, selecting and manipulating target genes is often desired (Nomaguchi et al. 2018) and a target organelle with genes that affect growth and lipid features of diatoms is the mitochondrion. Diatom mitochondria are a conserved organelle with an important role, especially in coupling with the chloroplast, to shuttle intracellular carbon for metabolism (Bailleul et al. 2015). The mitochondrion accounts for the ecological success and adaptation strategies of diatoms, and mitochondrial studies are an indispensable aspect to understanding diatom physiology and evolution (Jallet et al. 2020). Improving our ability to manipulate diatom growth in culture requires a deeper understanding of their genetic components, especially the role of mitochondria that may render diatoms superior in culture over other algae. Evaluating more diatom species and exploring their mitogenomes will improve our ability to manipulate diatom growth in culture while increasing data on diatom mitochondria, provide new insights into the molecular framework of diatoms, and help generate stronger phylogenetic relationships for more robust evolutionary analyses (Cochrane et al. 2020; Falciatore et al. 2020).

Mitochondrial genomes of algae, especially of diatoms, have intermediate mitogenome sizes, between the larger vascular plant mitochondria and smaller animal mitochondrial sizes (Lynch et al. 2006). Though generally smaller than plant
mitochondria, diatom genomes vary in size and intricacy. Studies on the characteristics of diatom mitogenomes have revealed much architectural similarities and differences as well as shed light on evolutionary relationships among diatom genera and the organisms involved in diatom evolution outside of the stramenopiles (Guillory et al. 2018; Pogoda et al. 2019). The size and complexity among diatom mitogenomes largely stem from ancestral and/or laterally transferred genomic artefacts or retrotransposons including repeat regions and pervasive introns (Ehara et al. 2000; Kamikawa et al. 2009; Oudot-Le Secq and Green 2011; Villain et al. 2017;). The genetic features that make diatom mitogenomes unique are often elucidated through comparative genomics (Ravin et al. 2010).

Comparative genomics is a useful tool to unravel the potential genetic basis for the observed diversity and success among species (Kroth et al. 2008; Blaby-Haas and Merchant 2019). Comparative analyses are helpful in uncovering differences in photosynthesis, carbon fixation, carbon storage, and lipid metabolic processes and even physiology among diatoms (Montsant et al. 2005; Hildebrand et al. 2013; Ogura et al. 2018). The mitogenome is an especially useful genomic compartment in characterizing and expanding the understanding for success of key diatom species (Pogoda et al. 2019). There is a general lack of comparative genomic studies, in context of comparing either oleaginous or alkaliphilic diatoms. Much of what is known about diatom genomic architecture come from studies on marine diatoms like *Phaeodactylum tricornutum*, *Skeletonema marinoi* (An et al. 2017). Sequencing the genomes of diatoms that use alternate habitats like alkaliphiles or extremophiles potentially expands our understanding of diatom mitogenomes. There also exists a large gap in the numbers of sequenced
diatom mitogenomes in relation to the proposed number of diatom species (Pierella Karlusich et al. 2020). Additional genomes of diatoms that have yet to be sequenced, including that of sister species, and the consequent comparative analyses resulting are required to posit the potential differences in metabolism involved in growth and lipid metabolism.

Mitogenome comparisons can be crucial for phylogenetic analyses particularly at the family level (Gastineau et al. 2018). Discerning the relationship among diatoms with higher certainty, especially the pennate diatoms, is ongoing and enabled by continuous genome sequencing (Tang and Bi 2016). Additional genomes provide computational power for resolving taxonomic queries such as the phylogenetic relationships, answered through comparative means. Comparative genomics between species is equally an important feature in population genetics for estimating relative organelle substitution rates, or frequencies at which SNPs (single nucleotide polymorphism), retroelements, repeats, or varying genes are gained or lost (Pogoda et al. 2009). Comparative analyses between species using multiple organelle genomes or distinct and separate species can yield important information on diatom genome structure and evolution (Smith 2015). Comparative studies of diatom sister species within the genera *Halamphora* and *Nitzschia* have already indicated substantial genome rearrangements and variation within each respective genus (Pogoda et al. 2019) even extending beyond the mitogenome [chloroplast] (Hamsher et al. 2019). Though much has been revealed through genome comparisons, microbial eukaryote mitogenomes generally lack investigation compared to higher taxonomic groups and warrants an expansion in existing mitogenome databases to fill taxonomic gaps that may elucidate patterns of diatom organelle genome evolution.
Where intrageneric comparisons are sought, more comprehensive, comparative, and hypothesis-driven analyses of new and existing genomes are necessary (Smith 2016) for more powerful phylogenetic and evolutionary interpretations (Ravin et al. 2010; Medlin & Desdevises 2016, 2020; Crowell et al. 2018; Guillory et al. 2018; Hamsher et al. 2019; Pogoda et al. 2019).

Mitochondria are involved in diatom lipid metabolism and carbon pathways, and therefore comparative mitogenome studies are a step in determining the genetic underpinnings that render some diatoms superior in cultivation. The diatom genus *Fistulifera* contains species with simultaneous high growth and lipid production, characteristics of potential strains for algae cultivation (Matsumoto et al. 2014; Berthold et al. 2020). *Fistulifera* species have also shown high productivity in marine, brackish, or alkaline waters (Satoh et al. 2013; Sato et al. 2014; Berthold et al. 2020) which makes *Fistulifera* a flexible and adaptable organism for algal cultivation. Genome comparisons among *Fistulifera* species and with other diatom genera can yield important information to the productivity and growth success of this small raphid diatom clade. To explore the mitogenome differences between prospective cultivation strains, comparative mitogenome analyses of sister *Fistulifera* species was performed. The marine *F. solaris* and the alkaliphilic *F. alcalina* were compared to provide insights into their success in light of physiological differences. The mitogenomes of *Fistulifera alcalina* is herein described and compared to extend data on diatom mitochondria and provide new insights into molecular framework of diatoms.
2. MATERIALS AND METHODS

2.1 Algal culture and DNA extraction

An axenic culture of *Fistulifera alcalina* (BLCC-F88) was grown in a 125ml flask with 50 µmol m⁻² s⁻¹ fluorescent light at 120rpm. *Fistulifera alcalina* was cultured in modified Zarrouk’s media (MZ10) adjusted using NaOH (5.2 g L⁻¹; pH=10), NaSiO₃, Gambourg’s vitamin mix, and vitamin B12 (Berthold et al. 2020). Prior to DNA extraction, the alga was harvested using a microcentrifuge tube and fresh biomass was washed five times with pure sterile water to remove bicarbonates and media contaminants. DNA was extracted using the DNeasy plant minikit (Qiagen, Düsseldorf, Germany) and purified using a QIAquick PCR purification kit (Qiagen).

2.2 Mitogenome sequencing, assembly, annotation, and alignment

Genome sequencing was carried out first by preparing libraries using the Illumina TruSeq Library Construction Kit followed by generation of 2 × 150-nucleotide paired-end reads with Illumina HiSeq instrument (Novogene Co., Ltd, Beijing, China). Trimming was done using FastP (4) and quality subsequently checked using FastQC v 0.11.9 (5). Mitogenome was assembled by first mapping raw reads onto the *Fistulifera solaris* (KT363689) reference mitogenome using BWA mem (Burrows-Wheeler Aligner) and extracting the unmapped reads. Reads were then assembled and used as a reference sequence for the comparison to the *de novo* assembly of the reads using SPADEs (v3.14.1; Bankevich et al. 2012). The more accurate assembly was further processed
using into one contig on Geneious Prime (v2020.2.4; Biomatters, Auckland, New Zealand).

Mitogenome annotation and gene prediction was carried out on Geneious built in “ORF prediction” and “annotate from database” features using a database of 22 closely related diatom mitogenomes, including *F. solaris*. Repeat regions within genomes were evaluated using “repeat finder” plugin tool on Geneious. Variant calling was carried out by first mapping raw reads onto the *F. solaris* reference genome followed by using FreeBayes (v1.1.0) and built-in Geneious variant calling using 30X coverage as a cutoff to call the SNPs. Both analyses were compared and summed into a congruent file.

Mitogenome structural illustrations from resulting annotations were constructed using GenomeDraw (Greiner et al. 2019) and further processed on Adobe Illustrator (v10.15).

Pairwise mitogenome alignment between *Fistulifera alcalina* and *F. solaris* was carried out in Geneious (using 93% similarity cutoff). Whole genome alignment was also carried out using *F. alcalina* along with 7 other phylogenetically closely related diatom species using Mauve progressive alignment tool (V1.1.1; Darling et al. 2004).

### 2.4 Phylogenetic analysis

A concatenated phylogenetic analysis of 14 protein-coding genes (*cox1-3, cob, nad1-7, nad4L, rps5-7, rrs, and rrl*) was carried out using *Fistulifera alcalina* and 21 available diatom mitochondrial genomes listed here along with their GenBank accession numbers, *Asterionella formosa* KY021079; *Berkeleya fennica* KM88661; *Cylindrotheca closterium* MG271845; *Didymosphenia geminata* KX889125; *Entomoneis* sp.
MF997419; Eunotia naegelli NC_037987; Fistulifera solaris KT263689; Halamphora calidilacuna MF997424; Halamphora coffeaeformis NC_037727; Haslea nusantara MH681882; Navicula ramosissima KX343079; Nitzschia alba MF997422; Phaeodactylum tricornutum NC_016739; Proschkinia sp. str. SZCZR1824 MH800316; Psammoneis japonica MG148339; Pseudo-nitzschia multiseries NC_027265; Skeletonema marinoi KT874463; Surirella sp. MF997423; Thalassiosira pseudonana NC_007405; Toxarium undulatum MG271847; Ulnaria acus NC_013710; with Phytophthora phaseoli HM590418 as an outgroup. Sequences pulled from NCBI and genes were concatenated and aligned using MAFFT alignment tool (v1.4.0; Biomatters Ltd). Resulting alignment was masked using 15% gap value and used to construct phylogenetic trees with RAxML on XSEDE (v7.2.7), through CIPRES (v.3.3). Consensus tree was visualized on iTOL (Letunic and Bork 2019; v5.7; http://itol.embl.de/) and edited on Adobe Illustrator (v10.15).

3. RESULTS AND DISCUSSION

3.1 Mitogenome evaluation and comparative genomics

From a total of 7,393,417 Illumina paired-end reads, 28,889 were assembled de novo into one contig (N50 of 26,334). Complete mitochondrial genome analyses of Fistulifera alcalina revealed a circular molecule of 37,055 bps and G+C content of 28.2% (Fig. 1). Fistulifera alcalina mitogenome encodes for a total of 78 genes including 2 rRNAs, 36 tRNAs, and 4 ORFs and 35 protein-coding genes (Table 1). Comparatively,
*Fistulifera solaris* and *F. alcalina* both share identical numbers of rsRNAs (2) and unidentified ORFs (orf51, orf66, orf154, orf251).
Figure 1 Mitochondrial genome structure of *Fistulifera alcalina* and *F. solaris*. 
Table 1 Features of sequenced diatom mitochondrial genomes

<table>
<thead>
<tr>
<th></th>
<th>Fistulifera alcalina</th>
<th>Fistulifera solaris</th>
<th>Berkeleya fennica</th>
<th>Proschkinia sp. SZCZR1824</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estimated genome size (bp)</td>
<td>37,055</td>
<td>39,468</td>
<td>35,509</td>
<td>48,863</td>
</tr>
<tr>
<td>GC content (%)</td>
<td>28.2</td>
<td>28.1</td>
<td>29.7</td>
<td>29.6</td>
</tr>
<tr>
<td>Total genes</td>
<td>78</td>
<td>63 [78]</td>
<td>63</td>
<td>57</td>
</tr>
<tr>
<td>Predicted protein-coding genes</td>
<td>36</td>
<td>36</td>
<td>36</td>
<td>33</td>
</tr>
<tr>
<td>tRNA</td>
<td>36</td>
<td>25 [36]</td>
<td>25</td>
<td>22</td>
</tr>
<tr>
<td>rRNA</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2 [4]</td>
</tr>
<tr>
<td>Coding DNA</td>
<td>38,982</td>
<td>33,266 [37,525]</td>
<td>32610</td>
<td>30,740</td>
</tr>
<tr>
<td>Non-Coding DNA</td>
<td>7,190</td>
<td>6,210 [4,217]</td>
<td>2899</td>
<td>18,123</td>
</tr>
<tr>
<td>% Non-coding DNA</td>
<td>18.4</td>
<td>15.7 [11.2]</td>
<td>8.2</td>
<td>37.1</td>
</tr>
<tr>
<td>Genbank</td>
<td>-</td>
<td>KT363689</td>
<td>NC026126</td>
<td>MH800316</td>
</tr>
<tr>
<td>References</td>
<td>This research</td>
<td>Tang &amp; Bi 2016</td>
<td>An et al. 2014</td>
<td>Gastineau et al. 2018</td>
</tr>
</tbody>
</table>

Values within [ ] represent data derived from re-analysis of genomic data in this work. [Coding DNA= rRNA+tRNA+CDS (coding+intronic)]

Pairwise mitogenome alignment of *Fistulifera alcalina* and *F. solaris* demonstrated 83% similarity through 33,307 matches and 6,817 differences in amino acid residues. Mapping of raw paired-end reads from *F. alcalina* revealed coverage between 40-1,687X for coding regions, while the coverage over the repeat region of *F. solaris* was at 10X or mainly without coverage or reads. Variant analysis confirmed 3,469 SNPs supported by coverage of 30X or higher. From the SNP analysis (Table 2), *F. alcalina* genome content contained a total of 124 indels (57 deletions and 67 insertions) with 533 substitutions compared to the reference sequence *F. solaris*. There
was a total of 1,961 transitions with 851 transversion events, with a high rate of transitions (Ts/Tv=2.0). The rate of Ts/Tv in *F. alcalina* is close but higher than the value of 1.6 reported by Rastogi et al. (2020) involving several clades of *Phaeodactylum tricornutum*.

**Table 2** Summary of SNPs and variants calculated from *Fistulifera alcalina* reads using *F. solaris* (KT363689) as a reference. Data based on 30X coverage and above.

<table>
<thead>
<tr>
<th></th>
<th>Count</th>
<th>Length range (bp)</th>
<th>Amino Acid Change (%)</th>
<th>Coding (%)</th>
<th>Noncoding (%)</th>
<th>Gene CDS with most effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNPs</td>
<td>3,469</td>
<td>-</td>
<td>28.1</td>
<td>85.9</td>
<td>14.1</td>
<td>-</td>
</tr>
<tr>
<td>Insertions</td>
<td>67</td>
<td>1-2</td>
<td>4.7</td>
<td>43.3</td>
<td>56.7</td>
<td><em>nad2</em></td>
</tr>
<tr>
<td>Deletions</td>
<td>57</td>
<td>1-13</td>
<td>5.2</td>
<td>35.1</td>
<td>64.9</td>
<td><em>nad2</em></td>
</tr>
<tr>
<td>Substitutions</td>
<td>533</td>
<td>1-13</td>
<td>59.1</td>
<td>76.0</td>
<td>24.0</td>
<td><em>nad2</em></td>
</tr>
<tr>
<td>Transitions</td>
<td>1,961</td>
<td>1</td>
<td>20.7</td>
<td>93.2</td>
<td>6.8</td>
<td><em>nad2</em></td>
</tr>
<tr>
<td>Transversions</td>
<td>851</td>
<td>1</td>
<td>29.0</td>
<td>82.0</td>
<td>18.0</td>
<td><em>nad2</em></td>
</tr>
</tbody>
</table>

Most SNPs that occurred throughout the mitogenome were significantly lower in the regions pertaining to the conserved *rrs* and *rrl* rRNA gene CDS, but especially highest for the *nad2* CDS. The *nad2* region, responsible for the NADH dehydrogenase subunit 2 protein, had the highest occurring variants including indels, substitutions, and transitions and transversions (Table 2). The *nad2* protein is a core subunit within the NADH complex involved in the respiratory or electron transport chain. The *nad2* gene of dinoflagellate endosymbionts were also reported to undergo extreme genetic makeover within the *nad2* CDS (Imanian et al. 2012).
Within SNPs, the most common was substitutions to the adenine residues (829 in total) versus the least altered residue of cytosine (571 in total). Within substitutive nucleotide SNPs pairs, the most common was substitutions of guanine and cytosine residues (total of 1186) versus substitutions of thymine and adenine (total of 1120). Though SNPs were abundant between sister species, gene block organization or locally collinear block (LCBs) retained complete synteny as revealed by one whole gene block between the two *Fistulifera* genome sequences (Fig. 1).

Besides synteny, other elemental features of genomes are retroelements or the inclusion of repeat regions. Retrotransposons such as introns are common features of diatom mitochondrial genomes and can account for large portions of genetic data (Pogoda et al. 2019). In this study, *F. alcalina* did not contain group I or II introns within the coding regions of the mitogenomes, similar to *F. solaris* (Tang and Bi 2015) and other diatoms such as *Nitzschia palea* (Crowell et al. 2018). The diatoms *Halamphora calidilacuna*, *H. coffeaeformis*, *Phaeodactylum tricornutum*, and *Proschkinia* all contain one or several introns throughout several genes including *cox1*, *cob*, *rrl* and *rrs*. Introns interrupt transcription and make the genes they disrupt impractical universal priming regions, especially the *cox1* (Pogoda et al. 2019), and oftentimes their source is unknown, and perhaps the question now can be why certain species of diatoms abstain from pervasive introns. Other contemporary thoughts impose intron selectivity within host organelle or host suppression of retroelements (Lambowitz and Zimmerly 2004). Organisms with small genomes and high mutation rates have been suggested to be prone to error correction (Lynch 2007), and in this light, the *F. alcalina* genome is constrained by its small size where errors and mutations would prove deleterious.
Repeat regions are other genetic features described from diatom genomes that are thought to support replication in higher organisms but lack general sequence or structure conservation in diatoms (Villain et al. 2017; Ravin et al. 2010). Repeat regions can either be present (Asterionella formosa) or absent (Berkeleya fennica) from a diatom mitogenome. The analyses demonstrated a small 23bp repeat region within F. alcalina, repH, with 91% identity (2 bp difference) to a motif found in the large repetitive region of Asterionella formosa (Villain et al 2017). Fistulifera solaris alternatively demonstrates 7 repeats of 2 motifs, with 188 and 122 pb in length, in the noncoding region of its mitogenome. The difference in mitogenome genome lengths (39 vs. 37 kb) between Fistulifera species could be a consequence of reduced non-coding region in F. alcalina or the inability for small reads technology such as Illumina, to cover more complex genomic regions containing repeats (Villain et al. 2017; An et al. 2016). However, with strong support from the high sequencing coverage on either side of the deletion within F. alcalina compared to F. solaris, the lack of reads within this deleted region, and together with the paired end reads spanning this region, machine error can be ruled out and a reduced mitogenome size in F. alcalina can be recognized. Reduction in mitogenome size can be a result of streamlining within F. alcalina in stable waters outside of oligotrophic waters, where Lake Okeechobee offers this alkaliophilic diatom a stable environment for success. The expansion in genome size, mainly through the introduction of repeat regions and introns, is well documented in diatoms including Asterionella formosa, Cylindrotheca closterium, Navicula ramosissima, P. tricornutum, Psammoreis japonica, and Thalassiosira pseudonana. The lack of both large repeat regions and introns, the high variation and SNPs between sister species but with retainment of gene
identity with synteny, and a reduction in overall genome size in comparison to a sister taxon potentially indicates a targeted reduction in genome size. This idea may be fortified with sequencing and annotation of the mitogenomes of additional *F. alcalina* isolates in order to comparatively suggest reduction in genome size.

### 3.2 Phylogenetic Analyses

Maximum likelihood phylogenetic analysis using sequences from 14 concatenated conserved mitochondrial genes (Fig. 2) demonstrated strong support for a large clade containing the genera *Fistulifera, Proschkinia, Berkeleya, Phaeodactylum, Didymosphenia*, and *Halamphora* (bootstrap, B.S.: 100). Within this clade, there was strong support for the delimitation of *Fistulifera* sister species *F. alcalina* and *F. solaris* (B.S.:100). The large clade entailing the 6 closely related diatom genera was also similarly, but not identically recovered in other phylogenetic analyses using mitochondrial, (Crowell et al. 2018; Gastineau et al. 2018; Pogoda et al. 2019), plastidial (Theriot et al. 2015), mitochondrial introns distribution (Guillory et al. 2018), and a mix of plastid and nuclear encoded genes (Theriot et al. 2010). The phylogenetic tree of 23 diatom species using 14 concatenated genes and one outgroup was used here to show the relationship of *F. alcalina* among the large diatom clade indicated. The phylogenetic placement of *F. alcalina* in the larger context of diatom phylogenetic classification (CMB hypothesis: Coscinodiscophyceae, Mediophyceae, Bacillariophyceae) requires larger taxon sampling, additional outgroups, and multiple representatives of those outgroups (Medlin and Desdevises 2020), an objective and devotion beyond the scope this paper.
Figure 2 Maximum likelihood phylogenetic tree of 14 mitochondrial concatenated genes using *Fistulifera alcalina* and 21 diatom mitogenomes with *Phytophthora phaseoli* (HM590418) as outgroup. Values presented at nodes are cut off at 50%.

To investigate the relationship between the genera within the well-supported clade that includes *F. alcalina*, a whole genome alignment demonstrated up to 7 conserved gene blocks of 1kb or greater length, among *Fistulifera*, *Proschkinia*, *Berkeleya*, *Phaeodactylum*, *Didymosphenia*, and *Halamphora* (Fig. 3). The most noticeable genome reorganization among the 7 diatom species is the displacement of the gene block containing nad11, nad4l, atp9, rpl16, rps3 (* in Fig. 3) in *Fistulifera* species as opposed to the remaining species. The synteny among these strains proved variable, though these changes suggest no overall change in function of the genome (Pogoda et al. 2019).
4. CONCLUSION

Sequencing more diatom species and exploring their mitogenomes expand data on diatom mitochondria and provide new insights into the molecular framework of diatoms. Mitochondrial studies, especially comparative analyses, are essential in understanding distinctions between sister taxa and diatom physiology and evolution. From the comparative mitogenome analyses of *Fistulifera* species, many SNPs were observed especially on the *nad2* gene, as well as nearly 2kb of sequence data absent from the non-coding region of *F. alcalina* in comparison to *F. solaris*. The substitution rate between *F. alcalina* and *F. solaris* was a little higher than that of *Phaeodactylum* species. *Fistulifera*
*alcalina* exhibits small cellular morphology in comparison to its sister species, which is also reflected in the smaller mitochondrial genome. Taken altogether, the size and genetic differences, especially reconfiguration of *nad2*, in mitogenomes between sister species, suggests *F. alcalina* is streamlining the mitochondria. Without large repeat region nor introns, *F. alcalina* appears to assume no tendency towards genome expansion.

The results presented here demonstrate how disparate diatom sister species genome can be, even when analyzing phylogenetically closely related taxa. Revealing substitution rates between sister diatom species is essential to arrive at better estimates for genome evolution. These results also provide insights into the comparative mitogenomics between sister taxa from disparate environments including saltwater and alkaline waters and analyses of two oleaginous diatom strains that are contenders for mass cultivation.

Bioprospecting alkaliphilic diatoms for the purpose of high growth in biotechnological applications could be a possible route as they are highly evolved to adapt to a specific environment whilst still holding true to their most characteristic lipid producing qualities. Since *F. alcalina* is an alkaliphilic diatom with a streamlined mitogenome, it would be helpful to apply this line of questioning to other alkaliphilic diatoms. Streamlined genomes are especially useful as they are easier to engineer than those with duplications, long repetitive sequence, and interruptive retroelements. Future work should focus on sequencing genomes of additional *Fistulifera* species and other oleaginous diatoms to further explore the connection between reduction in mitochondrial
genome size and the potential role it plays in determining the optimal environment and suitability of diatoms for cultivation.

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GENERAL CONCLUSIONS

The qualities that make diatoms ideal growth and lipid models and contenders for large scale cultivation are not completely understood. To improve our understanding of the drivers of diatom productivity this dissertation sought to explore: 1) what stimulates diatom successful growth in the environment and in culture, 2) diatom biodiversity especially at the species level, 3) algal physiology in the context of how diatom productivity may be enhanced by bacteria, and 4) the biological basis for the extraordinary growth rates of diatoms and their success in alkaline environments.

In an effort to locate oleaginous, alkaline-tolerant and/or alkaliphilic algae, the first goal of this dissertation was to bioprospect native algae from an alkaline Lake Okeechobee using classical microbiological methods of selection and isolation. Isolated strains of algae were assessed for tolerance to moderate to high alkaline treatments. By analyzing several different algal strains, a diatom of the genus *Fistulifera*, demonstrated suitable features for cultivation including alkaliphilic growth, with a fast-doubling rate and moderate lipid productivity.

The diatom isolate was subsequently characterized by growth experiments to evaluate the alkaliphilic, lipid and growth productivity potential for large-scale cultivation. Besides assessing algae physiological constraints, characterizing algae on a species level is a crucial aspect in biology since no two species are alike, and different algal species can widely diverge in physiology. Physiological experiments demonstrated a diatom with a propensity for alkalizing media with a combined high growth rate and lipid content. The next step was to describe this diatom in a molecular phylogenetic
context. The candidate diatom was formally described using the polyphasic approach of morphological scrutiny and molecular phylogenetics including multiple gene markers (18S rRNA and rbcL) to properly designate this unique species. The novel diatom species was then typified in accordance with the ICN (International Code of Nomenclature for algae, fungi and plants (Turland et al. 2018).

Since *Fistulifera alcalina* demonstrated preference for alkaline media with moderate lipid productivity, *F. alcalina* was used to investigate the effects of bacteria on diatom growth and/or lipid content in co-culture. To construct bacteria co-cultures, native bacteria found in the phycosphere of diatoms isolated from Lake Okeechobee and the water column were used. Co-cultures of *F. alcalina* with 22 bacterial isolates demonstrated either increase in growth rate or lipid content, but not the two simultaneously. From 22 isolated bacterial strains, one strain, *Bacillus horikoshii*, was capable of stimulating the specific growth of *F. alcalina*.

To further investigate *Fistulifera alcalina* and the diatom’s propensity for high growth rates in high alkaline media, the final goal of this dissertation was to use next generation sequencing and explore the diatom mitogenome and compare mitogenomes of sister *Fistulifera* species including *F. solaris*. Exploring genomes and using sequenced genomes in comparative analyses is a powerful method of pushing our understanding of diatom genetic architecture, phylogenetic placement, and organelle evolution. Using comparative genomics between the sister *Fistulifera* species indicated high mutation rates compared to other diatom species, and that *F. alcalina* retained a smaller, streamlined mitochondria. These results not only shed light onto diatom mitochondrial phylogeny, but also have implications on the role of genetic features such as streamlining in the context
of diatom evolutionary success in alkaline habitats. These results also have implications on the diatom strains potentially chosen for their improved growth rates and have implications towards demonstrating molecular differences between species in an evolutionary context.

By investigating the effects of bacterial presence on the novel diatom *Fistulifera alcalina*, this dissertation unifies classical methods of microbiological selection and isolation, phycological use of botanical codes and morphological validations using modern microscopic analyses, together with molecular phylogenetics and modern next generation sequencing in describing a novel diatom species, *F. alcalina*, and applying the alga in bacterial co-culture to stimulate productivity. There are many future prospects stemming from this research including overarching phylogenetic questions involving the description of the seemingly novel *Fistulifera* sp. BLCC-F141 and F144. By characterizing the novel diversity within this genus and sequencing the genomes, additional genomic comparative studies between all *Fistulifera* species including additional organelles such as the plastid and nucleus can be attempted. Comparing a genus with such diversity can be discern substitution rates and evolutionary patterns present in diatoms of variable environments. Lake Okeechobee is core genomic source for this diverse genus of particularly small diatoms, a sort of meeting place where all species coexist.
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