MALDI-TOF MS as a Rapid Characterization Tool for Economically-Relevant Microalgae

by

Duane Barbano

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved April 2016 by the
Graduate Supervisory Committee:

Todd Sandrin, Chair
Andrew Webber
Thomas Dempster

ARIZONA STATE UNIVERSITY

May 2016

ABSTRACT

The ability of microalgae to be mass cultivated and harvested for production of pharmaceuticals, nutraceuticals, and biofuels has made microalgae a focal point of scientific investigation. However, negative impacts on production are essentially inevitable due to the open design of many microalgae mass culture systems. This challenge generates a need for the consistent monitoring of microalgae cultures for health and the presence of contaminants, predators, and competitors. The techniques for monitoring microalgae cultures are generally time-intensive, labor-intensive, and expensive. The scope of this work was to evaluate the use of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) as a viable alternative for the characterization of microalgae cultures. The studies presented here evaluated whether MALDI-TOF MS can be used to: 1) differentiate microalgae at the species and strain levels, 2) characterize simple mixtures of microalgae, 3) detect changes in a single microalgae culture over time, and 4) characterize growth phases of microalgae cultures. This research required the development of a MALDI-TOF MS microalgae analysis protocol for organism characterization. The results yielded in this research showed that MALDI-TOF MS was just as accurate, if not more so, than molecular techniques for the identification of microalgae at the species and strain levels during its logarithmic growth phase. Additionally, results suggest that MALDI-TOF MS is sensitive enough to characterize simple mixtures and detect changes in cultures over time. The data presented here suggests the next logical step is the development of protocols for the near-real time health monitoring of microalgae cultures and detection of contaminants using MALDI-TOF MS.

ACKNOWLEDGMENTS

Multiple people assisted me throughout the course of my research and their aid is greatly appreciated. I would like to take the time to thank each of them for everything they contributed to my work and my success.

First and foremost, I would like to express my immense gratitude to my committee chair Dr. Todd Sandrin. Thank you for teaching me the skills I need to perform highly-relevant research and acting as my research mentor throughout my time at Arizona State University. You helped keep me on track and helped me overcome the obstacles I faced in my work. I would like to thank Dr. Thomas Dempster for providing me with the skills, materials, and knowledge required for my work to succeed. I would like to thank Dr. Andrew Webber for invaluable feedback on my thesis work and providing feedback on my progress. I would also like to extend my thanks to the Arizona Center for Algae Technology and Innovation and its members for all the assistance they provided me during my research.

I would like to thank my husband, Dustin Robertson, for providing me with the emotional advice and support I needed to complete my graduate thesis and research projects. Lastly, I would also like to thank my grandmother, Roberta LaMoureux, for providing me with the confidence, life skills, and love needed to be both happy and successful in life.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Background and Scope of Work

The mass culture of microalgae has gained popularity over the decades as a means for producing commodity and high-value products for multiple industries. In order to obtain cultures large enough for these industries the microalgae must be cultured in large ponds that are open or semi-open to the environment. The open configuration of the culture ponds allows for algae, bacteria, and zooplankton to contaminate ponds and produce negative effects [1] [2].

The introduction of an additional microalgae can result in the new organism out-competing and replacing the desired organism, resulting in a mass culture that does not yield the desired product. Commonly, the introduction of bacteria or planktonic predators results in total culture crashes that leave behind a dead and unusable culture. Currently, the characterization and identification of microalgae species relies heavily upon microscopic techniques, as does the monitoring of culture health (i.e. cell counts, cell density, presence of contaminants and predators, etc.).

Microscopy is one of the primary means for monitoring culture health. Additionally, cellular morphology analysis followed by 18s rDNA analysis is the gold standard for identification of microalgae down to the strain level. These techniques use a far-from-ideal amount of time and resources. Due to the rapidly expanding commercialization of microalgae and their products, newer methods for the rapid identification and characterization of organisms are needed.

Time and resources are minimized by using an alternative method for characterization and identification. Matrix-Assisted Laser Desorption/Ionization Time-

of-Flight Mass Spectrometry is a technique that analyzes cellular components in order to generate characteristic mass spectra. Previously, MALDI-TOF MS has been used to successfully characterize fungi [3] [4] [5] [6], bacteria [7], and simple polymicrobial mixtures [8]. The sample preparation method, MALDI-TOF process, and data analysis can all be completed in less than a day with highly accurate results. MALDI-TOF MS also provides users with a unique method for microalgae analysis by allowing a rapid view of the proteome, rather than the genome, of the organism.

The overarching goal of this work was to determine and evaluate the use of MALDI-TOF MS as a tool for the analysis of economically- and industrially -relevant microalgae. The scope of the work for the presented research required:

1. Developing a sample preparation and MALDI analysis protocol for microalgae.
    a. Minor changes to a common protein extraction method will yield usable proteins for MALDI-TOF MS analysis.
    b. Use of an automated data collection method starting at a low-laser intensity, collecting in 100 shot bursts, will yield high quality mass spectra.
2. Characterization of a collection of economically-relevant microalgae.
    a. MALDI-TOF MS analysis of a microalgae collection will allow for the construction of a spectra database for organism characterization and identification.

b. The creation of a spectra database of microalgae will facilitate the rapid analysis of previously uncharacterized species.

3. Characterization of simple mixtures consisting of eukaryotic organisms.
    a. MALDI analysis of artificial microalgae mixtures will allow detection of mixture and organism specific peaks.
    b. MALDI analysis of microalgae mixtures will afford taxonomic identification of the component members of the mixture.
4. Characterization of the growth curve dynamics of an economically-relevant microalgae species.
    a. MALDI analysis of an organism over time will allow users to determine physiological state of the organism.
    b. MALDI analysis of the growth curve of an organism will yield growth phase specific peaks.

Literature Review

MALDI-TOF MS Overview

The analysis of proteins and protein expression for the characterization and identification of organisms, known as proteomics, has become popular over the past few decades [9][10][11][12][13][14]. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a soft mass spectrometry technique that is used heavily in proteomics [15]. The spectra generated using MALDI-TOF MS are used as characteristic fingerprints for the identification of unknowns. Identification of unknowns is achieved by comparing the fingerprint spectra of the unknown to the

fingerprint spectra of known, reference spectra [16][17]. However, MALDI-TOF MS needs to be confirmed by genomic techniques in order to verify organism identifications. Genomics, in contrast to proteomics, is the study of genetic material. The most common genomic techniques used to verify identifications are 18s rDNA [18], repetitive sequence-based polymerase chain reaction (rep-PCR) [19], pulsed-field gel electrophoresis (PFGE) [20], and multilocus sequence typing (MLST) [21].

MALDI-TOF MS is a method for which an ion's mass-to-charge ratio (m:z) is determined by measuring the time it takes for the ion to travel a known distance. In order to ensure that all the ions have a standardized initial velocity, they travel through an electrical field of a pre-determined strength. While traveling through the time-of-flight tube, the ions' velocity along the set distance will change based on the weight of the ion. The heavier the ion, the slower that ion will travel and the higher its mass-to-charge ratio. The opposite is also true, the lighter the ion then the faster it will travel and the smaller its mass-to-charge ratio. A detector within the system allows the ions to be displayed as a mass spectrum (**Figure 1)** on a computer [22].

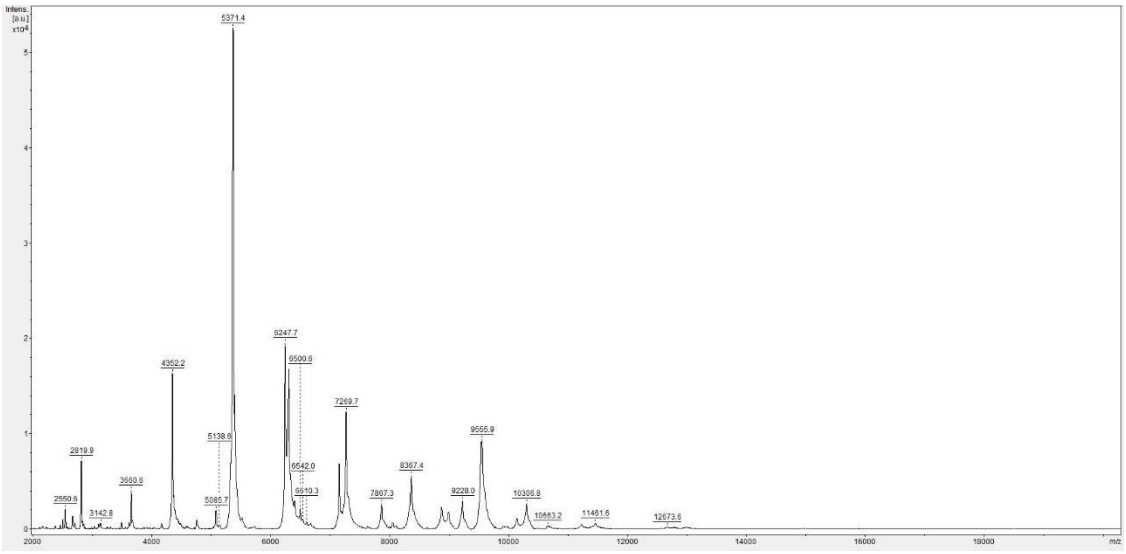

Figure 1. Example mass spectra of *Escherichia coli* bacteria.

Technology and Technique Overview

Development of the MALDI-TOF MS technology began with the combination of mass spectrometry and laser desorption techniques. Early work demonstrated that polar and nonvolatile organic molecules, derived from biological samples, could be analyzed by desorbing the molecules from a metallic surface [23]. Desorption from the surface was facilitated by a high-powered laser striking the bio-organic molecules. The laser implemented utilized a microsecond laser pulse that produced cations and fragments derived from the bio-organic molecules. The desorption application was further refined after the ideal laser wavelength was determined. Protocols using ultraviolet (UV) lasers with wavelengths of 266 nm and 355 nm were used to desorb amino acids and dipeptides from a metallic surface and generate characteristic spectra [24].

Protocols that utilized a chemical matrix to enhance desorption of non-volatile compounds quickly followed, which became the earliest methods for MALDI-TOF MS [23]. The matrices used for MALDI-TOF MS could be solid or liquid and were found to

resonate best with a laser wavelength of 266 nm [25]. The selection of a proper matrix led to decreases in unwanted molecule fragmentation [26]. The initial studies on MALDI-TOF MS were used to analyze compounds with relatively low molecular weights. Previous work demonstrated intact molecules and dimers produced from proteins larger than 10,000 daltons, but fragmented smaller proteins [27][28]. Following the work on an extended mass range, multiple studies that expounded on applications, protocols, and optimization emerged.

MALDI-TOF MS operates by firing a UV laser fired at a target plate that is coated in the organic molecule of interest (**Figure 2**). Once the laser strikes the molecule-matrix complex, the molecule is volatilized and launched into the time-of-flight (TOF) tube – this is the desorbing and ionization step. The materials then collide with a detector located at the end of the TOF tube and the data is relayed to a computer where mass spectra are generated. The spectra generated show the components of the material analyzed based on mass to charge ratios (m:z). The peaks are given arbitrary units (a.u.) to measure the intensities of molecules and fragments present [29]. It is important to note that the mass spectra generated are qualitative and not quantitative. MALDI-TOF MS does not allow users to determine the amount of an ion present within a sample.

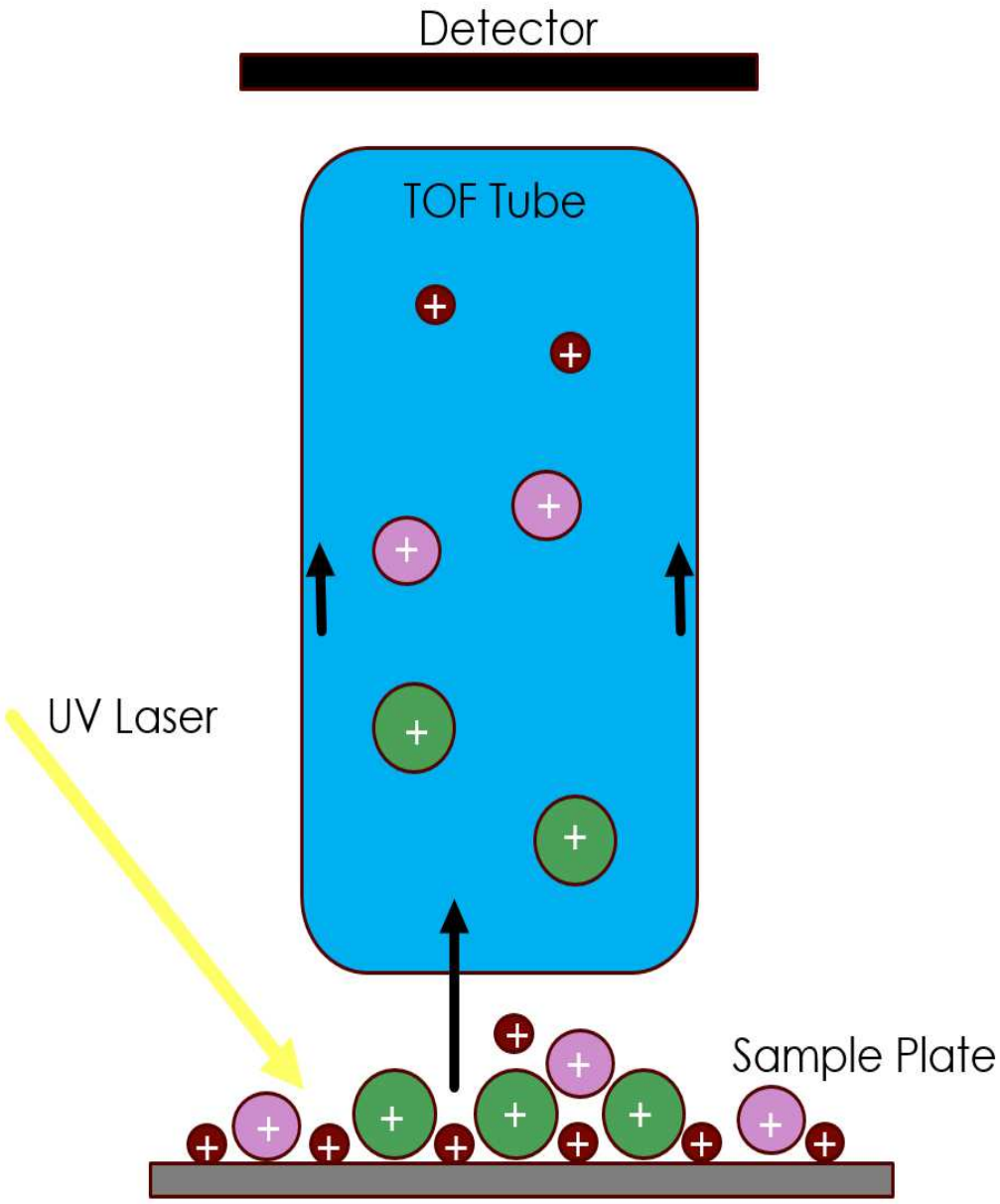

Figure 2. Schematic demonstrating the MALDI-TOF MS process.

Matrix-assisted laser desorption/ionization techniques have also been expanded to be repetitious in order to obtain more accurate results. MALDI can be tandem TOF (TOF-TOF) [30], tandem MS (MS-MS) [31], and tandem TOF-tandem MS (TOF-TOF MS-MS) [32]. In tandem TOF, multiple time-of-flight tubes are used in sequence. The

tandem MS makes use of quadrupole ion traps or fourier transform ion cyclotron resonance machines in order to analyze masses in timed steps. These tandem techniques are useful in analyzing fragments and precursor ions.

After expanding MALDI-TOF MS techniques to be repetitious, further advances have enabled the characterization for a plethora of organic molecules; including carbohydrates, nucleic acids, and biopolymers [33]. Interestingly, MALDI was shown to be effective in characterizing polymers reaching up to 300,000 daltons in size and also demonstrated that analysis time of bio-molecules could be completed in minutes [34]. After multiple successes, MALDI quickly became a popular analysis tool for proteomics. The process became popular in the characterization and identification of organisms at the genus, species, and strain-levels. Currently, MALDI-TOF MS has been used to characterize multiple organisms; including fungi [35][36][37][38], bacteria [39][40], microalgae [41][42][43], clinical samples[44], and simple polymicrobial mixtures [45][46].

Methods of Analysis

Two of the most common methods for sample preparation for organism analysis using MALDI-TOF include Intact Cell Mass Spectrometry (ICMS) and Protein Extraction Mass Spectrometry (PEMS). The ICMS process includes simply mixing matrix and deactivated cells, however it requires that the organism be left intact prior to placement on the target [47][48][49]. The PEMS requires that the organisms' cells be deactivated and then lysed by physical and chemical action prior to mixing the cellular extract with matrix and applying it to the target [50][51][52]. For both of the

aforementioned sample preparations, the final material to be placed on the MALDI target must either be mixed with matrix prior to placement or the material can be placed and then overlaid with matrix [53].

Once the sample has been prepared and the spectra have been generated, they are analyzed using one of two approaches: library-based methods or bioinformatics-enabled methods. In library-based approaches, the spectra of the unknown samples are compiled and compared to libraries or databases of well-characterized reference organisms [54][55]. The bioinformatics-based approach relies upon the identification of individualized peaks within an unknown organism's spectrum. Those peaks are characteristic of specific proteins and are compared to databases with genome sequence data in order to identify organisms [11][56]. Each approach has benefits and drawbacks. The library-based approach is best for organisms that have not yet been characterized or identified, but the drawback is that spectrum-reproducibility has the potential of being low. The bioinformatics-based method does not rely on highly reproducible spectra, but it is limited by the limited amount of genetic sequences of reference organisms [57].

Library-based approaches are used more commonly than bioinformatics-enabled approaches. The library-based approach is simple in that spectra from unknown organisms generated using MALDI-TOF MS are compared to databases of reference organisms. It is typically used to categorize, differentiate, and identify unknown organisms, however, in order to identify the organism, it must first be categorized and then differentiated [58].

The bioinformatics-enabled approach utilizes one of three strategies; intact biomarker identification, bottom-up analysis [59], or top-down analysis [31]. The intact biomarker approach requires that the spectra are run through a protein database, which allows for organism and strain-specific biomarkers to be identified. The bottom-up approach requires that cellular extracts be digested by enzymes and then the peptides are analyzed using MS-MS. The identified proteins then lead to organism identifications. In the final method, the top-down approach, MALDI-TOF-TOF is used to identify proteins that lead to organism identification [60] [31] [61] [62].

Software

Currently, one of the most commonly used software programs for analyzing MALDI-TOF mass spectra is the BioNumerics (version 7) software from Applied Maths. This software is used to build databases of organism spectra so that unknown organisms [17][50] [63] can be characterized and identified. Additionally, online databases may also be used for organism identification. The BioNumerics software provides users with copious amounts of algorithms for analyzing spectra. BioNumerics can be used to generate dendrograms, spectral images, pseudo-gels, similarity matrices, jackknife analyses, and multi-dimensional scaling analyses. All of these analyses are used to characterize and compare the unknown organism spectra to those of known reference organisms, they demonstrate the similarity, or differences, between organisms. In addition to BioNumerics, there are multiple other software packages available for mass spectra analysis. Software packages included, but not limited to; BioTyper [52][64][65],

SARAMIS [66][67][11], and MATLAB [68][69][70]. Many laboratories develop their own in-house software for database building and spectral analysis.

Microalgae

Description of Relevant Taxa

The term algae is used to refer to a large group of organisms that can be highly diverse in morphology and chemical processes. Algae can be unicellular, such as microalgae, or they can be multicellular, such as seaweed. Algae are primarily photoautotrophic and rely on photosynthesis in order to survive. Through photosynthesis, algae are able to take energy from sunlight in order to combine water, and carbon dioxide (CO_2) to generate algal biomass. Their processes of energy consumption are similar to terrestrial plants, but algae do not have the complex morphology observed in terrestrial plants [71]. Over the decades, the mass culture of algae has been driven by the demand of industry. Components of algal biomass have been found to be highly relevant to the pharmaceutical, nutraceutical, feedstock, fertilizer, food, and biofuel industries [72].

Algae can be found in both marine and freshwater ecosystems across the globe. Microalgae are found in both aquatic environments and they are difficult to observe without the use of a microscope, except for color when cultures are dense. The microalgae are of particular interest to industry because they can be easily cultured in a laboratory, photobioreactor, or terrestrial pond system within a relatively short span of time.

Types of Microalgae

Cyanobacteria – The cyanobacteria can be found in virtually all aquatic and terrestrial ecosystems, and have even been isolated in non-arable regions [73]. They are capable of photosynthesis and fixing CO_2 and they are also capable of nitrogen fixation [71][74].

Chlorophyceae – These organisms are members of the green algae and they receive their classification based on their chlorophyll a and b that gives them, their green color. These organisms are further classified by the extracellular theca and their telophase spindle [71]. Furthermore, the flagellar arrangement of the organisms can be used to classify the organisms as well. Currently, eight order of Chlorophyceae exist. Relevant members include; *Chlamydomonas*, *Scenedesmus*, and *Dunaliella* [74].

Bacillariophyceae – These organisms are commonly referred to as diatoms. The diatoms are unicellular algae, with some organisms having the ability to form colonies. The characteristic feature of the diatoms is the two-part silica-based cell wall called the fustule. The fustule can take on many geometries and produce multiple colors that provide characteristic cell walls that help in the identification of the organisms. Diatoms can be found in both freshwater and marine environments [75][71]. Important diatoms include; *Thalassiosira* and *Cyclotella* [74].

Chrysophyceae – The Chrysophyceae, or golden algae, are primarily freshwater organisms. The golden algae are characterized by their chemical make-up and

morphology [76][77]. The golden algae contain three separate chlorophylls; *a, c_1,* and c_2. Morphologically, they have two flagella that are oriented perpendicular to one-another where they attach to the cell. Interestingly, the shorter of the two flagella contains a single photoreceptor [71]. When these algae are exposed to adverse conditions, they will form statospores in order to survive. The organism, *Ochromonas,* is a relevant example of the golden algae [74].

Eustigmatophyceae – These organisms constitute a very small group of microalgae, but are quite economically significant. These organisms are unicellular with a coccoid cell morphology. The cell walls are characterized by their high polysaccharide content. The only chlorophyll they contain is type *a* [78]. These organisms have a yellow-green color that is derived from the chlorophyll and the accessory pigments, β-carotene and violaxanthin. Additionally, they have a single pair of flagella that attach at the apex of the cell [71]. Primarily, these organisms can be found in freshwater systems and soils. However, some species such as *Nannochloropsis* are found in marine environments [79][74].

Porphyridiophyceae – These red-colored algae are commonly found in soils and also on walls in humid environments. In order to mass culture these organisms, they must be cultured in marine media, as they are inhibited in freshwater media. These organisms can be filamentous or unicellular and often grows in layers that will appear blood-red. These organisms can be identified by their large, singular, stellate chloroplast. They will produce and excrete large amounts of

polysaccharides to form a capsule to protect the organism when conditions become unfavorable. These polysaccharides can be used for the production of ethanol. The *Porphyridium* genus is included within this class, along with *Rhodella* and *Rhodosorus* [71][74].

Trebouxiophyceae – The unicellular organisms are coccoid in their morphology and do not have flagella. They contain chlorophyll *a* and *b*, which gives them their green color. These organisms are of particular interest to the biotechnology field due to their ability to be easily grown *en masse* for a plethora of applications. Relevant member organisms include; *Chlorella*, *Parachlorella*, and *Prototheca* [71][74].

Chlorodendrophyceae – These chlorophyte algae are found primarily in marine environments. These organisms also have a theca outside of their cells that is formed by stellate scales. These organisms have four flagella that are attached within a pit in the theca of the organism and each flagellum is covered in hairs and scales. Relevant organisms include; *Tetraselmis*, *Platymonmas*, and *Prasinocladus* [71][74].

Applications

Microalgae have many industrial uses when grown in mass culture. Early on, microalgae were cultured for rotifer, fish, and livestock feed or feed additives [80][81]. However, the microalgae have been shown to yield high amounts of polysaccharides, fermentation products, fertilizers [81], and more importantly, neutraceuticals [80][81],

pharmaceuticals [81], and biofuels. The microalgae cultured will vary by product, as each microalgae has different ideal production properties [72].

Of particular interest is algae-derived biofuels. This interest came from the realization that reliance on fossil fuels, like oil and coal, has become highly unsustainable [82]. Furthermore, the combustion of fossil fuels results in harmful greenhouse gases being released into the atmosphere. These greenhouse gases pose immediate health threats to fauna and can result in negative changes in climate over time [83]. The mass culture of microalgae is an attractive crop for biofuel production because it is environmentally friendly, provides relief from fossil fuels, and does not detract from food crops [84].

The key greenhouse gases identified by the Environmental Protection Agency are carbon dioxide, methane, nitrous oxide, and fluorinated gases. Of these four gases carbon dioxide has the highest global emissions rate consisting of 76% of all gases. Of that, 65% is generated by fossil fuels and industrial practices. In 1900, the amount of carbon emitted was less than 1,000 million metric tons. In 2010, that number spiked to approximately 9,500 million metric tons and it continues to grow to this day. Shockingly, 90% of that increase has happened in the past 40 years [85].

Currently, fossil fuels are subject to highly variable availability and is in high demand which causes fuel prices to soar. The use of biofuels provides another method for meeting fuel demands and can help reduce fuel costs. The process of switching the

oils produced by algae to a functional biodiesel is simple through the use of a transesterification reaction. Transesterification is the process of exchanging an organic functional group on an ester with a functional group on an alcohol. The triglycerides are reacted with alcohol in order to produce glycerol and methyl esters. The methyl esters are used as biodiesel. The alcohol commonly used in this process is ethanol [86].

Culturing Techniques

The Food and Agriculture Organization (FAO) for the United States has a generalized set of requirements for the growth of microalgae. There are five basic requirements for culturing algae, those are: temperature control, salinity, light intensity, photoperiod control, and pH control. The generalized temperature range for microalgae is 16-27°C, with an optimal range of 18-24°C. The salinity range for microalgae is 12k ppm to 40k parts per thousand [87]. The salinity of the culture medium can vary greatly though, due to requirements specific to each microalgae species.

The light intensity required will change based on the volume and density of the culture. Generally, the range is 1,000 – 10,000 lux, with an optimal range of 2,500 – 5,000 lux. The photoperiod can either be 16 hours of light with 8 hours of darkness or 24 hours of light. The 16:8 light-dark ratio is more representative to light cycles utilized in the field, however in a controlled lab setting the 24-hour photo period results in much higher culture densities. Lastly, the advised pH range for algae culture is 7 - 9 [87].

Two very important growth requirements that were not mentioned by the FAO are agitation and gas-exchange. The microalgae are photosynthetic organisms, which require carbon dioxide in order to carry out their processes. Without agitation, the microalgae

would settle to the bottom of the container and growth-rate is reduced. Agitation is an additional benefit provided by methods of gas-exchange. Gas-exchange can be facilitated through shaking the cultures, introducing gases by a pump, or facilitating by a paddle [87].

Media selection for culturing microalgae depends solely on whether it is a freshwater, brackish, or marine strain. Marine species require media with higher salinities, freshwater species require low salinities, and brackish have salinities between the two. A basic marine medium that is used commonly in algae culture is 1% F/2 [88]. The media is composed of seawater (artificial seawater works as well), deionized water, nitrate-containing compounds, and phosphate-containing compounds. In addition, the medium requires the addition of essential vitamins and trace metals.

The freshwater medium commonly used for culturing is BG-11[89]. This medium has similar nitrate and phosphate containing compounds like 1% F/2. Additionally, BG-11 requires magnesium-, calcium-, citrate-, and carbonate-containing compounds. Lastly, BG-11 medium requires a specialized trace metals solution. The reason why 1% F/2 does not have as many components as BG-11 is that most of the required nutrients are already being provided by the seawater.

Mass Production

Photobioreactors are culturing vessels found at various scales for the mass production of microalgae. The vessel allows the algae culture to capture light and grow rapidly in a controlled or semi-controlled environment. Photobioreactors can either be open systems (exposed to the environment) or closed systems (closed off from the

environment) [90]. Open systems come in four major orientations, tanks, ponds, raceway ponds [91][92][93], and circular ponds [94]. The closed system photobioreactors are commonly tubular, flat-plate, or bags [86].

The simplest container to begin algae culturing is a small culture flask placed on a shaker under a light source. These culture flasks are primarily used to grow and maintain starter cultures of algae organisms. Once the culture is dense enough, it can be used to begin cultures in larger photobioreactors, such as culture tubes (**Figure 3)**. The culture tube photobioreactor has a smaller glass tube that runs the length of the culture tube so that air can be delivered into the culture and provide agitation and gas-exchange. The glass air tube runs through a rubber stopper that is used to close off the culture from the environment and there is an additional hole within the stopper to act as an exhaust. The exhaust is filled with sterile glass cotton to prevent contaminants from entering the culture. Rubber tubes are used to deliver air from the manifold to the culture tubes and a disc air filter is used in line to prevent contaminants from entering the culture from the air source.

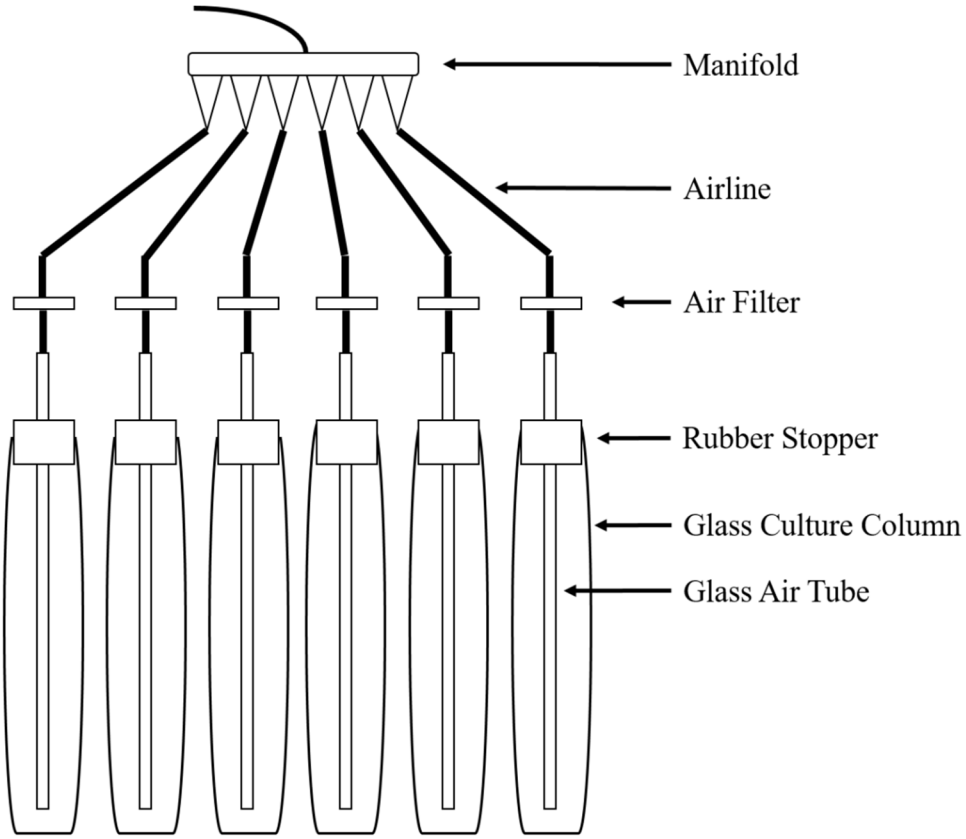

Figure 3. A schematic showing the basic culture tube photobioreactor.

An additional type of photobioreactor is the flat panel bioreactor (**Figure 4**). This type of bioreactor can be used within a laboratory or in the field. These are normally made from acrylic and designed to be thin so that light can penetrate the algae culture. A metal tube runs into the casing and curves along the bottom. The portion of the tube that runs along the bottom has holes drilled into it so that the air may escape into the culture so that agitation and gas-exchange may occur. The lid prevents contaminants from entering the culture and an air filter can be used to prevent contaminants from entering via the air flow.

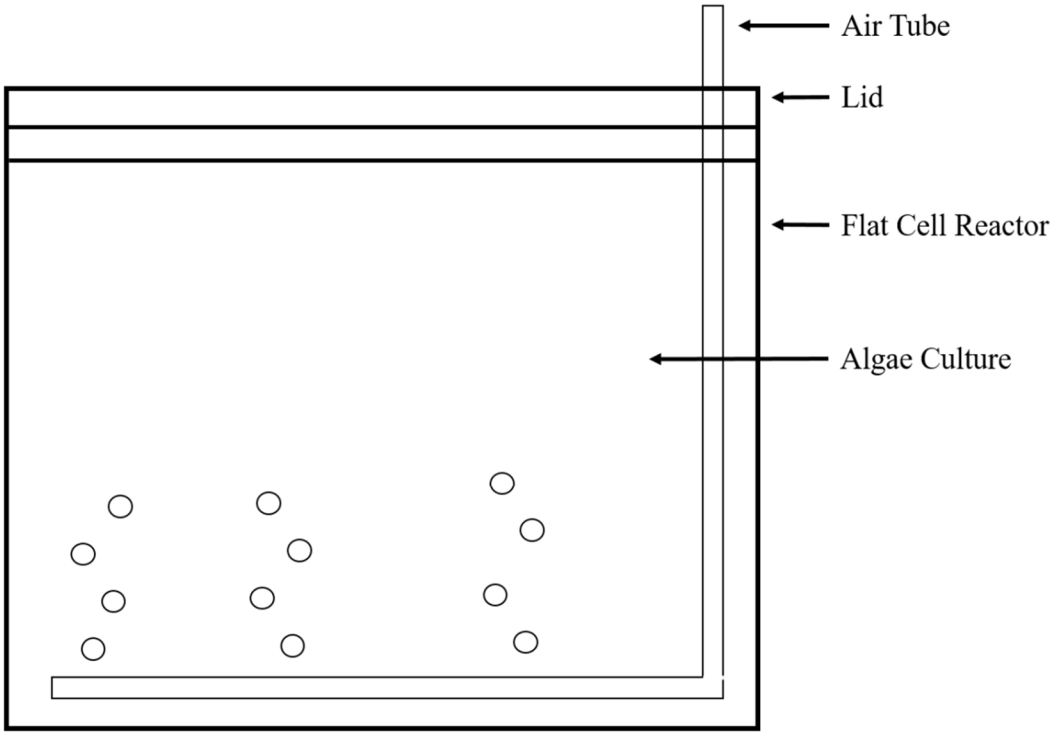

Figure 4. Schematic showing the basic set-up of a flat panel bioreactor.

The largest algae culture container is the pond. The pond can be a circular pond (**Figure 5**) or a raceway pond (**Figure 6**) [94]. The circular ponds are contained within berms oriented in a descending pattern. Algae culture will flow from the highest pond berm to the lowest pond berm. The descending flow provides the circular pond with gas-exchange and agitation. In the raceway pond, the culture is flowed through a "raceway" using a paddlewheel [93][94]. Baffles within the raceway are used to direct the flow of the algae culture. The paddlewheel is used to provide agitation and gas-exchange [86][95].

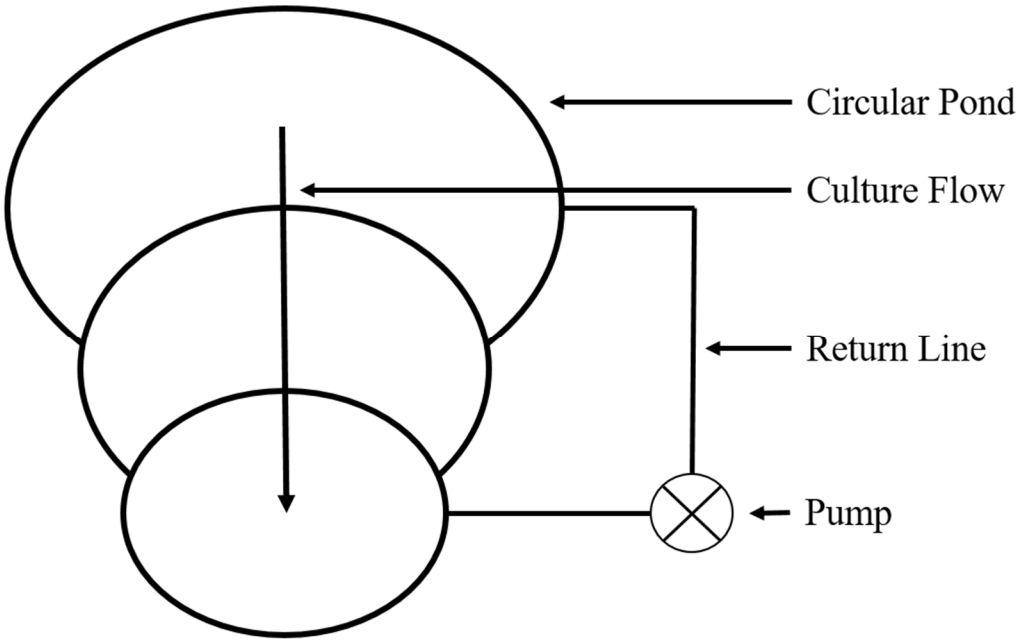

Figure 5. Circular pond schematic for algae mass culture.

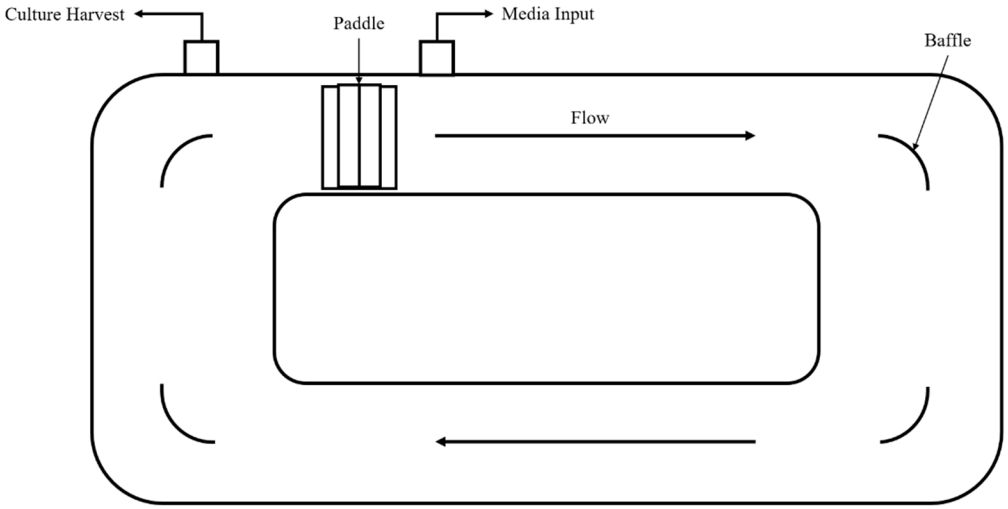

Figure 6. Schematic showing a raceway pond set-up.

Limitations

One of the largest limitations to products generated from algal biomass is the economic limitation of processes. The use of the term "processes' here is vague in that each process is highly detailed and specific, however the extraction of components from low-cost algae crops is reported as not economically balanced. Additionally, growth factors are also limiting factors for algae biomass production. Far-North and far-South regions of the globe have large variations in photoperiod and temperature and these two factors will have immense effects of algal growth. The depth of the algae culture also plays a large role in productivity. Cultures that are too deep will eventually become too dense for proper light penetration and productivity will be decreased. Many ponds and photobioreactors are restricted in their depth or width so that the light path can be optimized [96].

Open-pond cultures are also severely limited if monocultures are the desired end result. The only way to maintain the monoculture is by maintaining an extreme culturing environment. Extreme environments include those with high salinity, high nutritional status, and high alkalinity. So far, *Spirulina* and *Dunaliella* are the microalgae best suited for open-pond monoculture. Another limitations is the inability to properly sterilize culture containers for algae mass production, especially photobioreactors. The size of photobioreactors makes it difficult to sterilize them with heat or chemicals and then residual chemicals must be washed away with sterile water. Lastly, there is the high capital and operation costs of algae biomass production and the low-value crops produced [96].

Manuscript 1: Rapid Characterization of Microalgae and Microalgae Mixtures using Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Contributions of Author and Co-Authors

Author: Duane L. Barbano
Contributions: Conceived and designed the experiments, performed experiments, analyzed the data, and wrote the paper.

Co-author: Regina Diaz
Contributions: performed experiments, analyzed the data and wrote the paper.

Co-author: Lin Zhang
Contributions: Conceived and designed the experiments, performed experiments, analyzed the data, and wrote the paper.

Co-author: Todd Sandrin
Contributions: Conceived and designed the experiments, analyzed the data, and wrote the paper.

Co-author: Henri Gerken
Contributions: Conceived and designed the experiments, performed experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the paper.

Co-Author: Thomas Dempster
Contributions: Conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the paper.

Manuscript Information Page

Duane L. Barbano[1], Regina Diaz[1], Lin Zhang[2], Todd R. Sandrin[2*], Henri Gerken[3], and Thomas Dempster[3]
Journal Name: PLOSone
Status of Manuscript:
____Prepared for submission to a peer-reviewed journal
____Officially submitted to a peer-reviewed journal
____Accepted by a peer-reviewed journal
__x_Published in a peer-reviewed journal
Published online August 13th, 2015

[1]School of Life Sciences, Arizona State University
427 East Tyler Mall, Tempe, Arizona, 85281

[2]School of Mathematical and Natural Sciences, Arizona State University
MC 2352, P.O. Box 37100, Phoenix, Arizona, 85069

[3]Arizona Center for Algae Technology and Innovation, Arizona State University
7418 Innovation Way South, Building ISTB-3, Room 103, Mesa, Arizona, 85212

Abstract

Current molecular methods to characterize microalgae are time-intensive and expensive. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) may represent a rapid and economical alternative approach. The objectives of this study were to determine whether MALDI-TOF MS can be used to: 1) differentiate microalgae at the species and strain levels and 2) characterize simple microalgal mixtures. A common protein extraction sample preparation method was used to facilitate rapid mass spectrometry-based analysis of 31 microalgae. Each yielded spectra containing between 6 and 56 peaks in the m/z 2,000 to 20,000 range. The taxonomic resolution of this approach appeared higher than that of 18S rDNA sequence analysis. For example, two strains of *Scenedesmus acutus* differed only by two 18S rDNA nucleotides, but yielded distinct MALDI-TOF mass spectra. Mixtures of two and three microalgae yielded relatively complex spectra that contained peaks associated with members of each mixture. Interestingly, though, mixture-specific peaks were observed at m/z 11,048 and 11,230. Our results suggest that MALDI-TOF MS affords rapid characterization of individual microalgae and simple microalgal mixtures.

Keywords: Microalgae; MALDI-TOF MS; Discrimination; Mixtures; Strain; Economically Relevant; Mixture Characterization; Microalgae Analysis

Introduction

Microalgae have received considerable attention in science and industry as they can be cultivated and harvested for many products and co-products including biofuels and nutraceuticals [1]. Microalgae have different growth rates which are affected by a range of environmental factors such as nutrient availability and temperature. Those environmental factors need to be controlled in order to generate product, especially in large-scale biomass production [2]; however, the environmentally-exposed open pond system model leaves microalgae cultures susceptible to contamination by undesired microalgae that can out-compete the original microalga for resources, which can negatively affect production [2][3]. This shift in microalgae species can go unnoticed if the species are phenotypically similar. As a result, microalgae in mass-production systems need to be monitored regularly for contamination to avoid a decrease in productivity and catastrophic culture crashes.

Conventional techniques for microalgae identification include morphological analysis using bright field light microscopy and electron microscopy [4]. Complementary molecular techniques include multilocus sequence typing (MLST) [5], repetitive sequence-based polymerase chain reaction (rep-PCR) [6], 18S rDNA analysis [7], and pulsed-field gel electrophoresis (PFGE) [8]. In many instances, the use of these techniques requires amounts of time, labor, and resources that are impractical [9] for monitoring the health of microalgae ponds in near real-time.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a technique that has been shown capable of rapidly and reliably characterizing bacteria at the genus, species, and in some cases, strain levels [10] and is becoming more routine in use [11]. Most often, this is achieved by comparing mass spectra (i.e., fingerprints) acquired from crude protein extracts of unknown microorganisms to reference spectra in databases [12]. Furthermore, studies have shown that MALDI-TOF MS-based fingerprint methods may afford greater taxonomic resolution than traditional molecular techniques [9][10][13]. In addition to bacteria, MALDI-TOF MS has also been used to characterize fungi [14]-[17], viruses [18], and more recently to a considerably lesser extent, microalgae [4][5][19][20]. Nicolau and colleagues [4] obtained spectra of diatoms using MALDI-TOF MS and observed that culture age affected mass spectra. Von Bergen et al. [21] used MALDI-TOF MS to characterize five pathogenic species of *Prototheca*, and Wirth et al. [22][23] showed that optimization of downstream analyses such as self-organizing mapping (SOM portrait analysis) of spectra allowed MALDI-TOF MS to discriminate between harmless and pathogenic *Prototheca* species. Most recently, Emami et al. [20] obtained greater taxonomic resolution during characterization of 31 strains of *Dunaliella* sp. with MALDI TOF MS than with internal transcribed spacer (ITS) sequence analysis. Each of these studies suggests that MALDI-TOF MS has promise as a tool for the rapid characterization of diverse, economically-relevant microalgae [24][25].

To further explore the ability of MALDI-TOF MS to characterize microalgae, we focused on 31 algae representing 12 species. The specific objectives of this study were to

determine whether MALDI-TOF MS can be used: 1) for species-level differentiation of economically-relevant algae; 2) for strain-level characterization; and 3) to characterize simple mixtures of microalgae. A common protein extraction sample preparation method was used. Sequence (18S rDNA) analysis was performed on all microalgae to confirm their identity and to compare the taxonomic resolution afforded by this traditional approach to a MALDI-based approach. Finally, two model mixture systems containing two and three microalgae were examined. Our results suggest that MALDI-TOF MS affords rapid: 1) characterization of a diverse collection of microalgae, 2) discrimination between multiple strains within a single species, and 3) characterization of simple mixtures.

Materials and Methods

Reagents

MALDI matrix, α-cyano-4-hydroxycinnaminic acid (CHCA), and trifluoroacetic (TFA) acid were purchased from ACROS (Fair Lawn, NJ, USA). Acetonitrile (ACN) was purchased from Alfa Aesar (Ward Hill, MA, USA). MALDI calibrants (ACTH 1-17 (2,093.46 Da), ACTH 18-39 (2,464.19 Da), Insulin Oxidized B 3,494.65 Da), Insulin (5,730.61 Da), Cytochrome C (12,362.00 Da), and Apomyoglobin (16,952.30 Da)) and formic acid (FA) were purchased from Sigma (St. Louis, MO, USA). Ultrapure water was generated using a Milli-Q integral water purification system (Millipore Corporation, Billerica, MA, USA).

Microalgae Cultivation

Thirty-one microalgae representing 10 genera and 12 species were provided by the Arizona Center for Algae Technology and Innovation (AzCATI; http://www.AzCATI.com) (Table 1). Specifically, six of the genera were freshwater species (*Chlamydomonas*, *Chlorella*, *Parachlorella*, *Chromochloris*, *Desmodesmus* and *Scenedesmus*), and five were marine species (*Dunaliella, Chlorella, Tetraselmis*, *Nannochloropsis*, and *Porphyridium*). Five mL of BG-11 [26] for freshwater strains or F/2 [27] for marine strains were inoculated with a single colony of microalgae growing axenically on petri plates aseptically in a laminar flow hood. Microalgae samples were grown in 15 mL screwcap tubes with 75 µmol/m^2/s of cool white fluorescent lighting at 20°C for 3 weeks prior to analysis with MALDI-TOF MS.

Table 1. Microalgae species and strains used in this study.

Sample	Genus	Species	Strain	Medium	Genbank Accession Number
1	*Chlorella*	*vulgaris*	UTEX 395	Freshwater	KR904898
2	*Chlorella*	*vulgaris*	UTEX 259	Freshwater	KR904897
3	*Chlorella*	*vulgaris*	LRB-AZ 1201	Freshwater	KR904896
4	*Chlorella*	*sorokiniana*	UTEX 2714	Freshwater	LK021940.1
5	*Parachlorella*	*kessleri*	CBS 15-2069[1]	Freshwater	KR904906
6	*Chromochloris*	*zofingeinsis*	UTEX 32	Freshwater	KR904902
7	*Chromochloris*	*zofingeinsis*	LRB-AZ 701	Freshwater	KR904901
8	*Chlorella*	*sorokiniana*	UTEX 1230	Freshwater	KR904895
9	*Chlamydomonas*	*reinhardtii*	CC 849	Freshwater	KR904894
10	*Chlamydomonas*	*reinhardtii*	CBS 15-2030	Freshwater	KR904892
11	*Desmodesmus*	*abundans*	LRB-CO 801	Freshwater	KR904903
12	*Chlamydomonas*	*reinhardtii*	CBS 15-2280[2]	Freshwater	KR904893
13	*Scenedesmus*	*acutus*	LRB-AP 401	Marine	KR904911
14	*Scenedesmus*	*acutus*	LRB-AZ 414	Marine	KR904912
15	*Dunaliella*	*salina*	CBS 15-2160	Marine	KR904904
16	*Nannochloropsis*	*gaditana*	CCMP 526	Marine	KF040086.1
17	*Nannochloropsis*	*gaditana*	CCMP 527	Marine	AF045038.1
18	*Nannochloropsis*	*salina*	CCMP 1776	Marine	KJ756828.1
19	*Nannochloropsis*	*salina*	CCMP 537	Marine	AF045049.1
20	*Nannochloropsis*	*granulata*	CCMP 529	Marine	U41092.1
21i	*Chlorella*	sp.	LRB-AZ 1221	Marine	KR904899
22	*Nannochloropsis*	*limnetica*	CCMP 505	Marine	U41050.1
23	*Nannochloropsis*	*granulata*	CCMP 525	Marine	AF045044.1
24	*Nannochloropsis*	*oceanica*	CCMP 531	Marine	U41094.1
25	*Nannochloropsis*	*oceanica*	IMET-1	Marine	KR904905
26	*Nannochloropsis*	*oceanica*	CCAP 849/10	Marine	KJ756836.1
27	*Porphyridium*	*purpureum*	CBS 15-3599	Marine	KR904907
28	*Porphyridium*	*purpureum*	LRB-OH 6101	Marine	KR904908
29	*Tetraselmis*	sp.	CBS 15-2475	Marine	KR904909
30	*Tetraselmis*	sp.	CBS 15-2610	Marine	KR904910
31	*Chlorella*	*vulgaris*	LRB-FL 1220	Marine	KR904900

[1] Carolina 152069 was identified by Carolina Biological as *Chlorella spp*.
[2] Carolina 152280 was identified by Carolina Biological as *Haematococcus spp*.

Sample Preparation for MALDI-TOF MS

A common, previously described protein extraction procedure was used as the basis for the sample preparation method used here [28]. One mL of cells at an optical density of 750 nm (OD_{750}) between 0.15 and 0.3 were washed with sterile milliQ-H_2O (mQ-H_2O) and then inactivated for 1 hour in 300 µL mQ-H_2O and 900 µL absolute

ethanol. Samples were then centrifuged at 10,000 x g for two minutes at room temperature. The supernatant was decanted, and the cells were resuspended in 1 ml mQ-H_2O, centrifuged at 10,000 x g for two minutes once more, and the supernatant was again decanted. FA and ACN were added to the resulting pellet. Equal volumes of FA and ACN were added in volumes necessary to normalize to an initial culture $OD_{750} = 0.8$. Pellets were vortexed vigorously. The samples were then centrifuged at 17,000 xg for five minutes at room temperature, and the supernatant was collected and used immediately for MALDI analysis. Triplicate 1-µL aliquots of each supernatant were plated onto a MSP 96 Polished Steel MALDI Target Plate (Bruker Daltonics, Billerica, MA, USA) and allowed to air dry. A CHCA matrix solution was prepared by mixing 25 µL 99.5% TFA, 500 µL ACN, 475 µL mQ-H_2O, and 15 mg CHCA. Each sample was covered with 1 µL CHCA matrix solution and allowed to air dry.

Mass Spectra Acquisition

A Bruker Microflex LRF MALDI-TOF MS (Bruker Daltonics) was used to acquire mass spectra. The spectrometer was equipped with a 337 nm nitrogen laser and controlled using FlexControl software (version 3.0; Bruker Daltonics). Mass spectra in the m/z 2,000 to 20,000 range were collected automatically in the positive linear mode. Ion source 1 was set to 20 kV, and ion source 2 was set to 18.15 kV with the lens set to 9.05 kV. Spectra for each sample were generated from 500 laser shots acquired in five 100 shot bursts. The laser frequency was set to 10 Hz. Spectra from each of the 100 shot bursts were included only if the following parameters were met: a base peak (i.e., the peak with the greatest intensity) signal-to-noise ratio (S:N) of 2 or greater, a peak width

of 10 m/z, a minimum intensity threshold of 100, and a maximal number of peaks of 500. Three replicate MALDI mass spectra were obtained per algal strain. Peak smoothing was performed using the Savitzky-Golay algorithm. Baseline subtraction was performed using the TopHat algorithm. Calibration of the mass spectrometer was performed using a protein calibrant mixture containing the proteins listed above. Peaks were identified using FlexAnalysis 3.0 software (Bruker Daltonics) and then transferred to a Microsoft Excel spreadsheet in which the average mass range and base peak signal-to-noise ratios were calculated to assess spectrum quality. Peaks were considered different if they varied by more than +/- 2 m/z.

MALDI-TOF MS Data Analysis

Additional analysis of spectra was performed using BioNumerics (v. 7; Applied Maths, Austin, TX, USA). Composite spectra (i.e., summary spectra) were created using data from all replicates to represent each of the 31 microalgae. A similarity threshold of 65% was used to ensure representation of each replicate spectrum in the summary spectra. Pseudo-gels were constructed to visualize the MALDI profiles for each microalga. Similarity was quantified using the Pearson correlation coefficient, and a dendrogram was generated by using the UPGMA method.

18S rDNA Sequence Analysis

The genomic 18S ribosomal DNA region of microalgae was amplified by colony PCR as described previously [29] using the 360FE: 5'-CGGAGARGGMGCMTGAGA-3' [30] forward and 26R-1: 5'-GTTAGTTTCTTTTCCTCCGC-3' [30] reverse primers. Following amplification, PCR products were electrophoresed on a 0.8% agarose gel and

purified using a Zymoclean DNA gel recovery kit (Zymo Research, Irvine, CA). Sequencing was performed at the ASU Core DNA sequencing facility on 20 ng of template using the primers 360FE and 1391RE: 5'-GGGCGGTGTGTACAARGRG-3' described previously [30] and the following primers constructed for this study: 18SF3 5'-GTCAGAGGTGAAATTCTTGG, 18SF4 5'-CGGCTTAATTTGACTCAACACGGG-3', 18SR2 5'-AAGAACGGCCATGCACCACCACCC-3', 18SR3 5'-CCCAACTTTCGTTCTTGATTAATG-3'.

Analysis of the 18S rDNA sequences for the 31 microalgae samples was performed using BioNumerics (v. 7; Applied Maths, Austin, TX, USA). The similarity coefficient was multiple-alignment based with a Kimura correction and 12% gap penalty. The dendrogram build method was neighbor joining. No out group was used in the construction of the dendrogram.

Characterization of mixtures of microalgae using MALDI-TOF MS

Two model mixture systems were constructed using samples composed of two or three microalgae cultures. The OD_{750} for each microalga was adjusted to 0.3 before mixing so that the microalgae would be represented equally in the mixture. The first mixed culture contained two microalgae, *Chlorella vulgaris* UTEX 395 and *Scenedesmus acutus* LRB-AP 401. To construct the mixture, 500 μL of each culture were added to a microcentrifuge tube to yield a 1 mL solution. The second mixed culture contained three microalgae: *C. vulgaris* UTEX 395, *S. acutus* LRB-AP 401, and *Chlorella sorokiniana* UTEX 1230. The 1 mL mixture was constructed using 333 μL of each microalga. Samples were prepared for MALDI analysis, and mass spectra were acquired using the

procedures described above. Peak lists of the three individual samples and the two mixtures were compared. Each peak was included in the peak list only if it was present in all replicates. Peak matching was performed to identify prominent peaks attributed to individual isolates and peaks that were mixture-specific.

Results and Discussion

MALDI-TOF Spectra of Microalgae

MALDI-TOF MS yielded unique spectra for each microalga in the collection. We examined a mass range of m/z 2,000-20,000. A recent study [19] utilized a slightly narrower (m/z 4,000-20,000) range when characterizing microalgae via MALDI. The broader mass range we employed contained peaks that appeared useful for microalgae characterization. For example, several peaks below m/z 4,000 were observed in the spectra of multiple genera including *Chlorella vulgaris* UTEX 395 (Fig 1a), *Porphyridium purpureum* LRB-OH 6101 (Fig 1b), *Tetraselmis sp.* CBS 15-2610 (Fig 1c), *Chlamydomonas reinhardtii* CC 849 (Fig 1d), and *Nannochloropsis oceanica* IMET-1 (Fig 1e).

Mass ranges and peak numbers varied among the spectra of microalgae examined here. Masses in the spectrum of *C. vulgaris* UTEX 395 ranged from m/z 2,044 to 10,685. Comparatively, spectra of *P. purpureum* LRB-OH 6101, *Tetraselmis sp.* CBS 15-2610, and *C. reinhardtii* CC 849 exhibited narrower mass ranges (as low as m/z 2,005 and up to m/z 8,701 with *Tetraselmis* sp.). Spectra of *N. oceanica* IMET-1 had the broadest mass range (m/z 2,087 to 13,265). Numbers of peaks for the samples described here ranged from 6 for *Chromochloris zofingeinsis* UTEX 32 up to 56 peaks for *Nannochloropsis*

granulata CCMP 525. These results are comparable to the work of Lee et al. [7], who used MALDI-TOF MS to characterize *Nannochloropsis granulata, Chlorella* sp., and *Dunaliella* sp. While spectra shown here are not identical to those described previously (e.g., Lee et al. [7] reported a broader mass range with *Chlorella* as well as a prominent peak near m/z 8,700 not observed in our spectra), both our work and that of Lee et al. [7] suggests that MALDI affords rapid and clear differentiation of diverse microalgae.

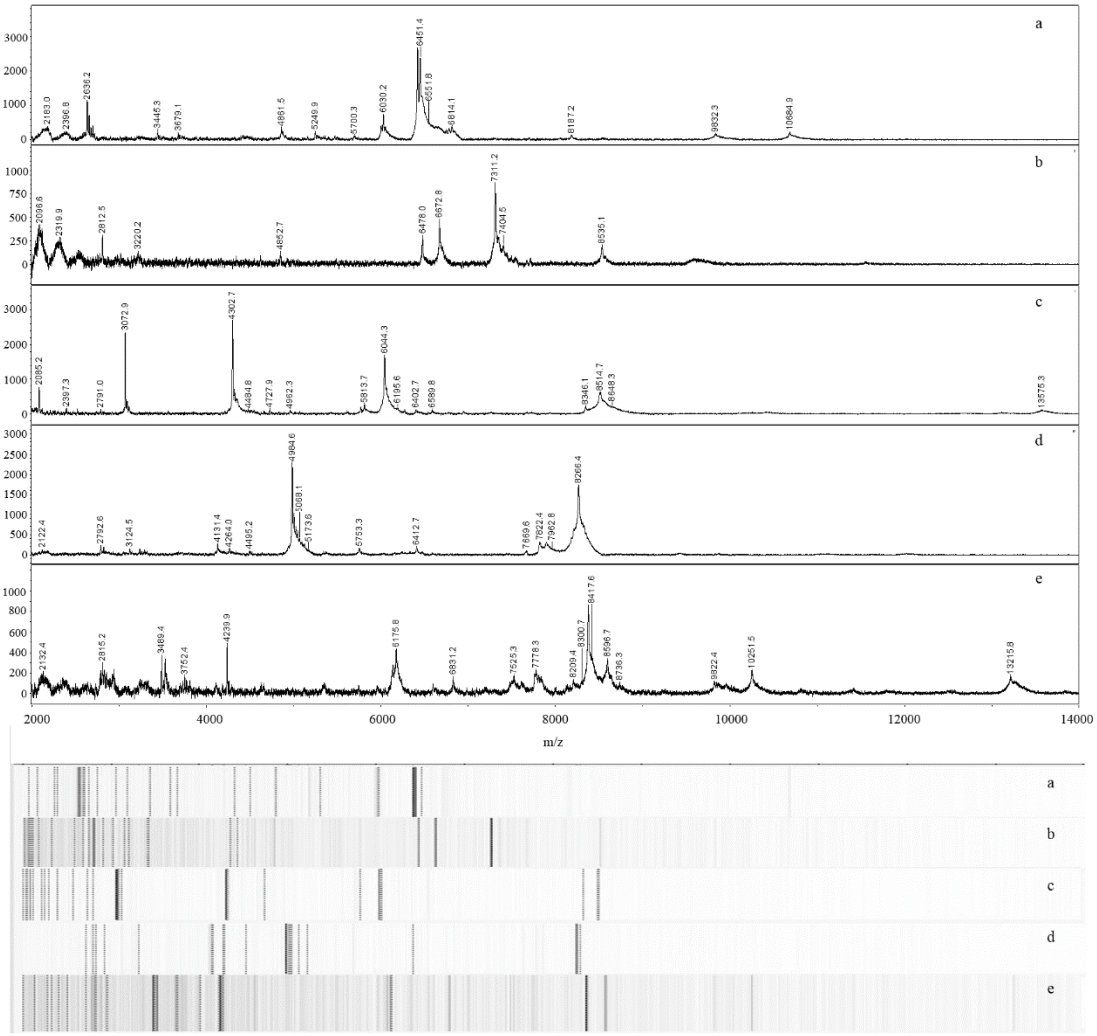

Fig 1. MALDI-TOF spectra and pseudo-gels of members of the five microalgae classes included in this study. Spectra and corresponding pseudo-gels of *Chlorella vulgaris* UTEX 395 (Trebouxiophyceae) (a), *Porphyridium purpureum* LRB-OH 6101

(Porphyridiophyceae) (b), *Tetraselmis spp.* CBS 15-2610 (Chlorodendrophyceae) (c), *Chlamydomonas reinhardtii* CC 849 (Chlorophyceae) (d), and *Nannochloropsis oceanica* IMET-1 (Eustigmatophyceae) (e).

Moving beyond species-level characterization, we examined the capability of MALDI to characterize microalgae at the strain-level. The works of Murugaiyan [23], von Bergen [21], and Wirth [22] using members of the genus *Prototheca* support the ability of MALDI to distinguish between strains of microalgae within the same species. Our data suggest that strain-level differentiation of members of the genus *Chlorella* is feasible. Mass spectra of three *Chlorella* strains (*C. vulgaris* UTEX 395, *C. vulgaris* UTEX 259, and *C. vulgaris* LRB-AZ 1201) are clearly distinct (Fig 2a-c). Spectra of all three microalgae exhibited similar mass ranges of m/z 2,182 – 10,685; 2,167 – 10,660; and 2,182 – 9,283, respectively; however, the spectra contained different base peaks at m/z 6,422; 2,637; and 2,517, respectively. These distinct spectra may be explained, in part, by the work of Gerken et al., who demonstrated previously that while the 18S rDNA sequences between 11 *C. vulgaris* strains were over 99% similar, the sensitivity of each strain to specific enzymes was remarkably distinct, indicating a highly variable cell wall composition among the various strains [29].

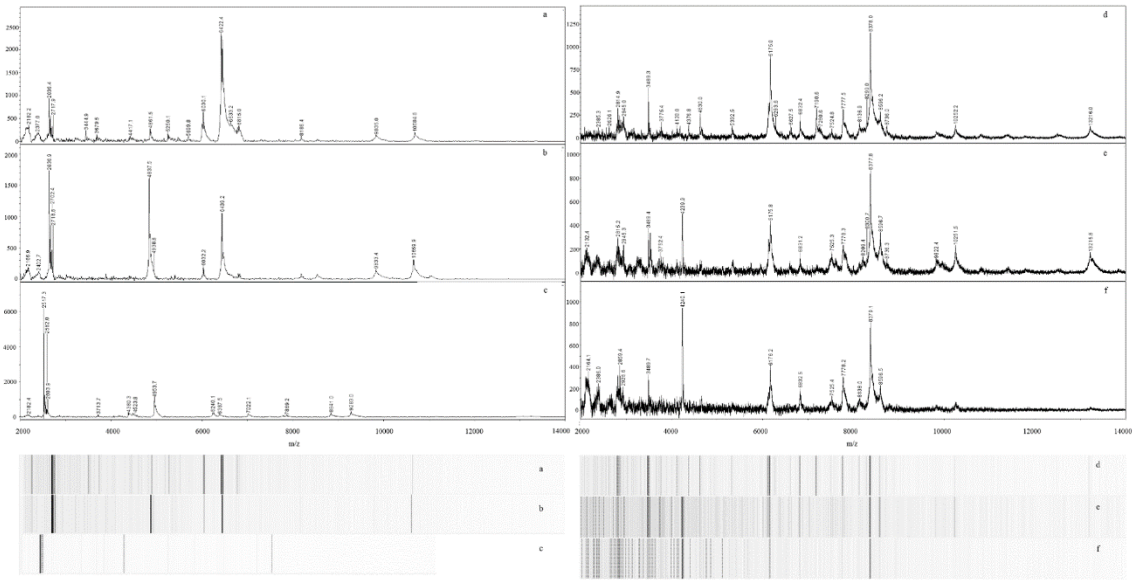

Fig 2. Representative MALDI-TOF spectra and pseudo-gels of microalgae strains of the same species. Three *C. vulgaris* spectra (a-c) exhibit strain-level differences: UTEX 395 (a), UTEX 259 (b), and LRB-AZ 1201 (c). Three *N. oceanica* spectra (d-f) also exhibit strain-level differences: CCMP 531 (d), IMET-1 (e), and CCAP 849/10 (f).

Similar to our results with *C. vulgaris*, spectra of three *Nannochloropsis* strains (*N. oceanica* CCMP 531, *N. oceanica* IMET-1, and *N. oceanica* CCAP 849/10) appeared to afford strain-level differentiation (Fig 2d-f). Spectra of all three of these strains contained a characteristic peak near m/z 8,378. The spectrum of strain CCAP 849/10 contained a different base peak (m/z 4,240) than the other two *N. oceanica* strains. Additional differences in peaks among spectra of these strains were observed (Fig. 2d-f).

Most recently, Emami et al. [20] have reported results similar to ours in which MALDI-TOF MS appeared to afford greater taxonomic resolution in microalgae than gene sequence-based methods. In particular, they were able to differentiate strains of *Dunaliella*. Similar to our work, they also used a mass range of m/z 2,000 to 20,000. Our work is comparable in size (i.e., number of isolates analyzed), but broader (i.e., focus

beyond a single genus) in taxonomy compared to the work of Emami et al. Interestingly, Emami et al. reported that whole cell-based sample preparation was necessary to yield useful spectra [20]. Our results, however, suggest that a relatively common, protein extraction-based approach to sample preparation is sufficient to produce MALDI spectra of microalgae that yield species- and strain-level characterization. While spectra we report here and those reported by Emami [20] for *C. vulgaris* are not identical, prominent peaks below m/z 3,000 are observed in spectra produced by both groups. Differences between spectra are likely related to different sample preparations, different strains, and differences in life stages used in each study.

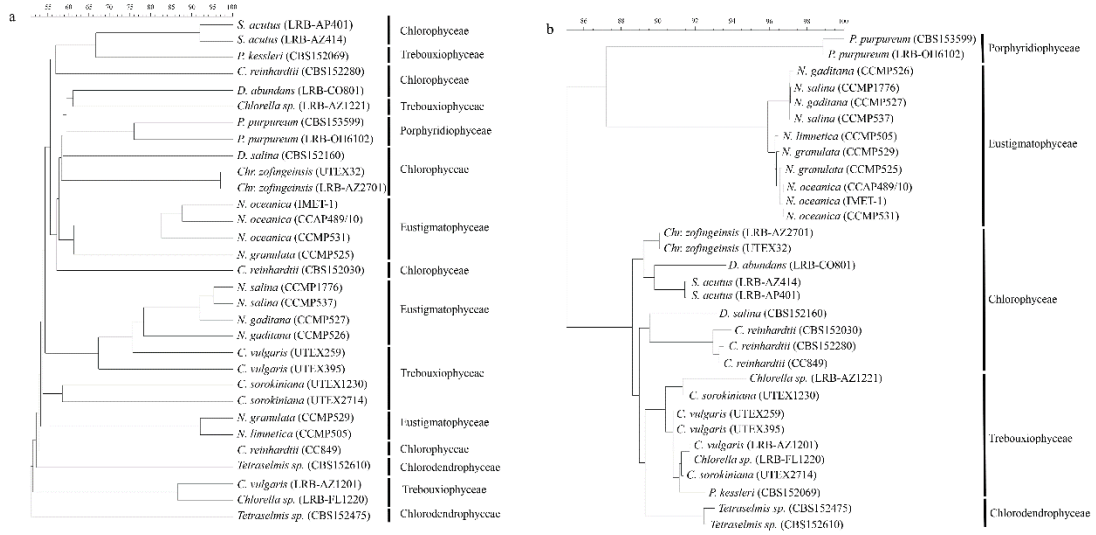

Fig 3. Similarity-based dendrograms representing spectra (a) and 18s rDNA sequences (b) of 31 microalgae. Class names are listed on the right to facilitate comparison between the two dendrograms. The 18s rDNA sequence-based dendrograms (b) shows grouping of the samples up to the class level. Spectra (a) were clustered using the UPGMA algorithm, while sequences (b) were clustered using the neighbor joining algorithm.

Comparison of 18S rDNA Sequence and MALDI-TOF Data

Differences observed in the spectra summarized above were reflected in the cluster analysis of spectra of all 31 microalgae examined here (Fig. 3a). Spectra of microalgae clearly separated at the species level. Separation of microalgae at the strain-level was also observed (Fig 3a).

We also performed 18S rDNA sequence analysis and compared it to the MALDI-TOF MS data. The 18S rDNA-based dendrogram (Fig. 3b) included five clades corresponding to five classes of microalgae represented in our collection. As expected, members of the same genus and species clustered together; however, at the strain level, the 18S rDNA sequence data did not afford clear separation of *Nannochloropsis salina* strains and *Scenedesmus acutus* strains. *N. salina* CCMP1776 and CCMP537 sequences had no differences in 18S nucleotide sequences; *S. acutus* LRB-AP 401 and LRP-AZ 414 differed by only 2 nucleotides. Additional DNA sequencing data of regions such as ITS-2 would be required to clearly differentiate these strains. In contrast, the MS-based dendrogram (Fig 3a) clearly separated nearly all strains examined including strains of *N. salina*, *S. acutus*, *C. vulgaris*, *C. reinhardtii*, *N. oceanica*, *N. gaditana*, and *P. purpureum*. The dendrogram based on the 18S rDNA data demonstrated a much higher degree of taxonomic organization (i.e., members of the same class clustered together) compared to the MS-based dendrogram as has been reported previously [7]. Similar to our results, Lee et al. [7] reported intermixing of taxonomically similar microalgae at the class-level in an MS-based dendrogram. Differences between MALDI- and 18S rDNA sequence-based dendrograms are reflective of the facts that: 1) 18S rDNA dendrograms are based

only on gene sequence data, while MALDI dendrograms and spectra contain proteome-level, gene expression-based data and 2) different clustering algorithms are routinely employed with each type of data (i.e., gene sequence data are typically clustered using the neighbor-joining algorithm [5], while MALDI spectra are often clustered using the Unweighted Pair Group Method with Arithmetic Average (UPGMA) algorithm [9]).

Mixture Analysis

Rapid detection of contaminating microalgae and deleterious community shifts are important during outdoor pond cultivation of microalgae biomass. For this reason, we attempted to use MALDI to characterize simple mixtures of microalgae. As has been reported frequently with MALDI analysis of bacterial mixtures, spectra of mixtures of microalgae contained many peaks originating from the individual microalgae composing the mixture [31] - [36]. The first mixture contained *C. vulgaris* UTEX 395 and *S. acutus* LRB-AP 401. Six prominent peaks from these two individual microalgae were observed in the spectrum of this mixture (Table 2; Fig. 4). The second mixture contained *C. vulgaris* UTEX 395, *S. acutus* LRB-AP 401, and *C. sorokiniana* UTEX 1230. Spectra from this mixture contained eight prominent peaks found in the spectra of the constituent three individual microalgae.

Not all peaks observed in spectra of individual microalgae were observed in the mixture spectra. Peaks present in the spectrum of *C. vulgaris* UTEX 395 dominated both mixture spectra (Table 2; Fig. 4). As previously postulated [31], ion suppression may account for underrepresentation of individual microalgae in the spectra of mixtures. Ion suppression results when one analyte suppresses appearance of another in a mass

spectrum due to: 1) the suppressing ion being present at a higher concentration than the suppressed ion and/or 2) the suppressed ion does not ionize as efficiently as the visible ion. We adjusted the OD_{750} of each microalgae sample to 0.3 before constructing the mixtures, but the *C. vulgaris* peaks remained the most prominent in the mixture spectra. It is possible that the *C. vulgaris* yielded more readily ionized proteins compared to *S. acutus*, but further work is warranted to further clarify mechanisms of peak suppression in microalgal mixtures.

Interestingly, spectra of both mixtures exhibited unique peaks at m/z 6,481; 11,048; and 11230. These three peaks appear to be mixture-specific as they do not appear in the spectra of the individual microalgae constituents. Similar results have been reported previously with bacterial mixtures [31], in which two mixture-specific peaks were observed in a mixed culture of *E. coli* and *S.* Typhimurium. The origin of these peaks and the mechanism of their formation is not clear, but may result from interactions between proteins (e.g., enzymes) associated with the individual cultures. Alternatively, interspecies interactions between the algae may have induced expression of proteins represented by these peaks. In either case, these mixture-specific peaks may provide information that is useful in the rapid characterization of algal mixtures and/or identification of contamination of algal cultures.

Table 2. Peaks observed in spectra of individual and simple mixtures.

m/z	*C. vulgaris* UTEX 395 (Sample 1)	*S. acutus* LRB-AP 401 (Sample 13)	*C. sorokiniana* UTEX 1230 (Sample 8)	Mixture 1 (Samples 1 & 13)	Mixture 2 (Samples 1, 8, &13)
2636	M			M	M
2712		M		M	M
2735		P			
2908			M		M
4608			M		M
6030	M			M	M
6422	M			M	M
6451	M			M	M
6481				S	S
10684	M			M	M
11048				S	S
11230				S	S

M = peak from individual microalga that was observed in one or both mixtures
P = peak from individual microalga that was not observed in either mixture
S = mixture-specific peak

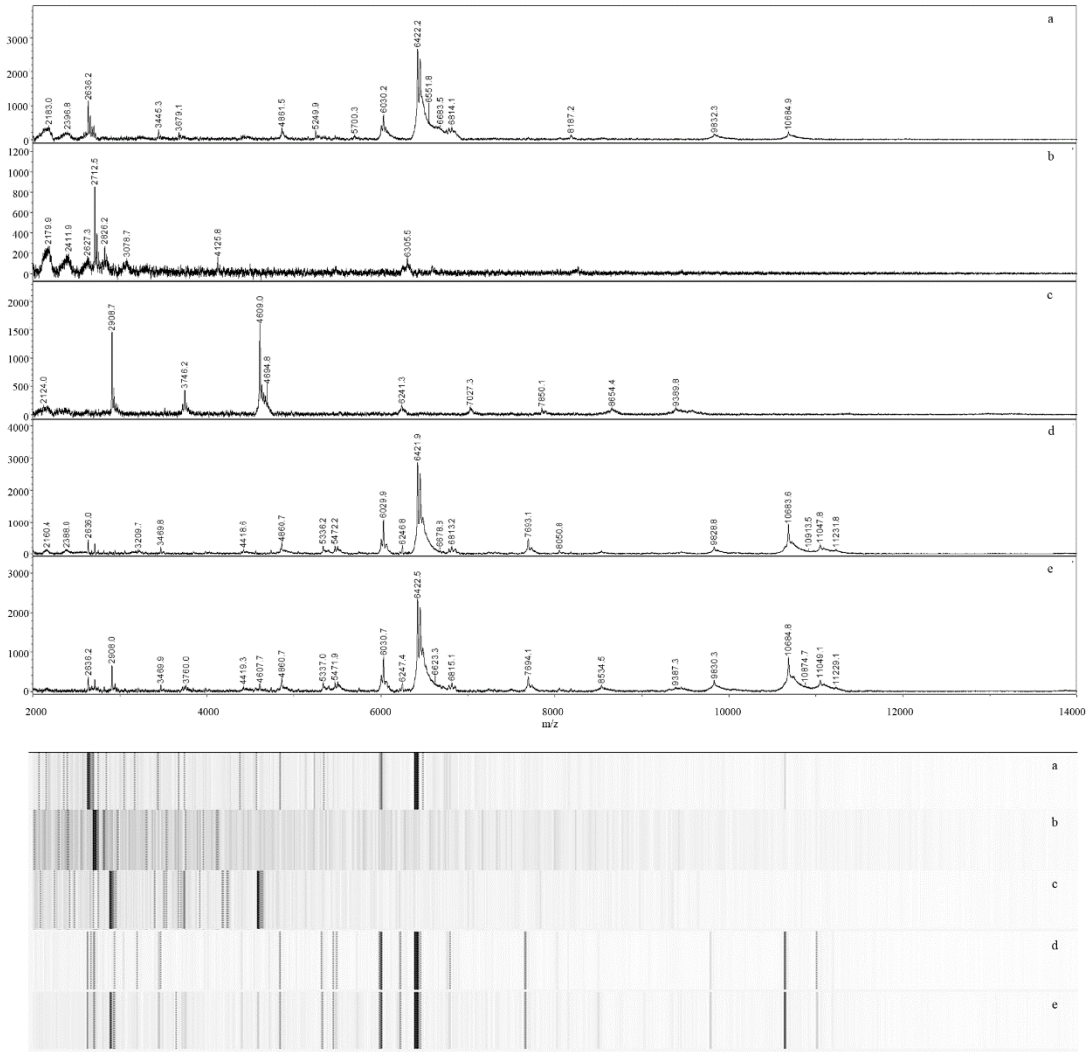

Fig 4. MALDI-TOF spectra and pseudo-gels of simple mixtures containing two or three individual microalgae. Representative spectra of samples of *Chlorella vulgaris* UTEX 395 (a), *Scenedesmus acutus* LRB-AP 401 (b), *Chlorella sorokiniana* UTEX 1230 (c), a mixture of *C. vulgaris* UTEX 395 and *S. acutus* LRB-AP 401 (d), and a mixture of all three microalgae (e).

Conclusions

Our results suggest that MALDI-TOF MS represents a rapid and effective alternative to conventional methods of characterizing microalgae. To our knowledge, this is the first report of the use of MALDI to characterize mixtures of microalgae (polycultures), which are gaining popularity within the microalgae production industry.

The taxonomic resolution of this rapid approach appears superior to conventional gene-sequencing based methods, as has been reported recently with *Dunaliella* [20]. Mixture-specific peaks were observed and may serve as biomarkers of contamination that allow producers to rapidly detect contamination events. Accordingly, MALDI-TOF MS has potential as a more rapid and economical means of monitoring the health and productivity of microalgae culture systems. For this reason, our current efforts include development of sample preparation and data analysis workflows that facilitate rapid analysis of more complex microalgal mixtures, including those that result from contamination events and predator introduction.

Acknowledgements

This work was supported by the Arizona Center for Algae Technology and Innovation, the ASU New College of Interdisciplinary Arts & Sciences, and ASU Lightworks.

References

1. Ho SH, Ye X, Hasunuma T, Chang JS, Kondo A (2012) Perspectives on engineering strategies for improving biofuel production from microalgae – a critical review. Biotechnol Adv 32: 1448-1459.

2. Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S (2010) Biofuels from algae: challenges and potential. Biofuels 1: 763-784.

3. Smith VH, Crews T (2014) Applying ecological principles of crop cultivation in large-scale algal biomass production. Algal Res 4: 23–34.

4. Nicolau A, Sequeira L, Santos C, Mota M (2014) Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) applied to diatom identification: influence of culturing age. Aquat Biol 20: 139-144.

5. Wei L, Xin Y, Wang D, Jing X, Zhou Q, Su X, et al. (2013) Nannochloropsis plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. BMC Genomics 14: 534-550.

6. Godhe A, Anderson DM, Rehnstam-Holm AS (2002) PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. Harmful Algae 1: 375-382.

7. Lee HW, Roh SW, Cho K, Kim KN, Cha IT, Yim KJ, et al. (2015) Phylogenetic analysis of microalgae based on highly abundant proteins using mass spectrometry. Talanta 132: 630-634.

8. Oh HS, Ah Kum K, Kim EC, Lee HJ, Choe KW, Oh MD (2008) Outbreak of Shewanella algae and *Shewanella putrefaciens* infections caused by a shared measuring cup in a general surgery unit in Korea. Infect Control Hosp Epidemiol 29: 742-748.

9. Giebel R, Worden C, Rust SM, Kleinheinz GT, Robbins M, et al. (2010) Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): applications and challenges. Adv Appl Microbiol 71: 149-184.

10. Sandrin TR, Goldstein JE, Schumaker S (2013) MALDI-TOF MS profiling of bacteria at the strain level: a review. Mass Spectrom Rev 32: 188-217.

11. Ng W (2013) Teaching microbial identification with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and bioinformatics tools. J Microbiol Biol Educ 14: 103-106

12. Zhang L, Vranckx K, Janssens K, Sandrin TR (2015) Use of MALDI-TOF mass spectrometry and a custom database to characterize bacteria indigenous to a unique cave environment (Kartchner Caverns, AZ, USA). J Vis Exp 95.

13. De Bruyne K, Slabbinck B, Waegeman W, Vauterin P, De Baets B, Vandamme P (2011) Bacterial species identification from MALDI-TOF mass spectra through data analysis and machine learning. Syst Appl Microbiol 34: 20-29.

14. Pavlovic M, Mewes A, Maggipinto M, Schmidt W, Messelhausser U, Balsliemke J, et al. (2014) MALDI-TOF MS based identification of food-borne yeast isolates. J Microbiol Methods 106: 123-128.

15. Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M (2013) Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF mass spectrometry. Med Mycol 51: 580-585.

16. Wieme AD, Spitaels F, Vandamme P, et al. (2014) Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a monitoring tool for in-house brewer's yeast contamination: a proof of concept. J Inst Brew 120: 438-443.

17. Deak E, Charlton CL, Bobenchik AM, Miller SA, Pollett S, McHardy IH, et al. (2015) Comparison of the Vitek MS and Bruker Microflex LT MALDI-TOF MS platforms for routine identification of commonly isolated bacteria and yeast in the clinical microbiology laboratory. Diagn Micr Infec Dis 81: 27-33.

18. Calderaro A, Arcangeletti MC, Rodighiero I, Buttrini M, Gorrini C, Motta F, et al. (2014) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification. Sci Rep 4.

19. Andrade LM, Mendes MA, Kowalski P, Nascimento CAO (2015) Comparative study of different matrix/solvent systems for the analysis of crude lyophilized microalgal preparations using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 29: 295-303.

20. Emami K, Hack E, Nelson A, Brain CM, Lyne FM, Mesbahi E, et al. (2015) Proteomic-based biotyping reveals hidden diversity within a microalgae culture collection: an example using *Dunaliella*. Sci Rep 5: 10036.

21. von Bergen M, Eidner MA, Schmidt F, Murugaiyan J,Wirth H, Binder H, et al. (2009) Identification of harmless and pathogenic algae of the genus Prototheca by MALDI-MS. Proteomics Clin Appl 3: 774-784.

22. Wirth H, von Bergen M, Murugaiyan J, Roesler U, Stokowy T, Binder H (2012) MALDI-typing of infectious algae of the genus Prototheca using SOM potraits. J Microbiol Methods 88: 83-97.

23. Murugaiyan J, Ahrholdt J, Kowbel V, Roesler U (2012) Establishment of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry database for rapid identification of pathogenic achlorophyllous green micro-algae of the genus *Prototheca*. Clin Microbiol Infec 18: 461-467.

24. Smith, VH (2014). Advances in algal biofuels and bioproducts. Industrial Biotechnology 10: 159–161.

25. Knoshaug EP, Darzins A (2011). Algal Biofuels: The Process. Chem Engin Prog 107: 37–47.

26. Stainer RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (Order Chroococcales). Bacteriol Rev 35: 171-205

27. Guillard RRL. Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH, editors. Culture of marine invertebrate animals. New York: Plenum Press; 1975. pp. 29-60.

28. Freiwald A, Sauer S (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc 4: 732-742.

29. Gerken H, Donohoe B, Knoshaug E (2013) Enzymatic cell wall degradation of *Chlorella vulgaris* and other microalgae for biofuels production. Planta 237: 239-253.
30. Dawson SC, Pace NR (2002) Novel kingdom-level eukaryotic diversity in anoxic environments. Proc Natl Acad Sci 99: 8324-8329.

31. Holland RD, Wilkes JG, Cooper WM, Alusta P, Williams A, Pearce B, et al. (2014) Thymol treatment of bacteria prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis aids in identifying certain bacteria at the subspecies level. Rapid Commun Mass Spectrom 28: 2617-2626.

32. Mandrell RE, Harden LA, Bates A, Miller WG, Haddon WF, Fagerquist CK (2005) Speciation of *Campylobacter coli*, *C. jejuni*, *C. helveticus*, *C. lari*, *C. sputorum*, and *C. upsaliensis* by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 71: 6292-6307.

33. Mahe P, Arsac M, Chatellier S, Monnin V, Perrot N, Mailler S, et al. (2014) Automatic identification of mixed bacterial species fingerprints in a MALDI-TOF mass-spectrum. Bioinformatics 30: 1280-1286.

34. Hsieh SY, Tseng CL, Lee YS, Kuo AJ, Sun CF, Lin YH, et al. (2008) Highly efficient classification and identification of human pathogenic bacteria by MALDI-TOF MS. Mol Cell Proteomics 7: 448-456.

35. Wahl KL, Wunschel SC, Jarman KH, Valentine NB, Petersen CE, Kingsley MT, et al. (2002) Analysis of microbial mixtures by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Anal Chem 74: 6191-6199.

36. Jarman KH, Cebula ST, Saenz AJ, Petersen CE, Valentine NB, Kingsley MT, et al. (2000) An algorithm for automated bacterial identification using matrix-assisted laser desorption/ionization mass spectrometry. Anal Chem 72: 1217-1223.

Manuscript 2: Growth Phase Affects MALDI-TOF Mass Spectra of *Chlorella vulgaris* Cultures.

Contributions of Author and Co-Authors

Author: Duane L. Barbano
Contributions: Conceived and designed the experiments, performed the experiments, analyzed the data, and wrote the paper.

Co-author: Todd R. Sandrin
Contributions: Conceived and designed the experiments, analyzed the data, and wrote the paper.

Co-author: Thomas Dempster
Contributions: Conceived and designed the experiments, analyzed the data, contributed reagents/materials/analysis tools, and wrote the paper.

Manuscript Information Page

Duane L. Barbano[1], Todd R. Sandrin[2*], and Thomas Dempster[3]
Journal Name:
Status of Manuscript:
__x_Prepared for submission to a peer-reviewed journal
____Officially submitted to a peer-reviewed journal
____Accepted by a peer-reviewed journal
____Published in a peer-reviewed journal
Published online August 13th, 2015

[1]School of Life Sciences, Arizona State University
427 East Tyler Mall, Tempe, Arizona, 85281

[2]School of Mathematical and Natural Sciences, Arizona State University
MC 2352, P.O. Box 37100, Phoenix, Arizona, 85069

[3]Arizona Center for Algae Technology and Innovation, Arizona State University
7418 Innovation Way South, Building ISTB-3, Room 103, Mesa, Arizona, 85212

Abstract

Current methods for monitoring microalgae growth over time are limited in terms of accuracy, time requirements, and cost. Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) may represent a viable alternative for monitoring microalgae growth. Growth of *Chlorella vulgaris* UTEX 395 was measured using optical density at 750 nm followed by MALDI-TOF mass spectrometry analysis every 24 hours. The overarching objective of this work was to determine if MALDI-TOF MS is sensitive enough to detect changes in a microalgae culture based on culture age. Rapid mass spectrometry based analysis of *Chlorella vulgaris* UTEX 395 was facilitated through the use of a common protein extraction method. Resulting spectra contained between 21 and 73 peaks within the m/z 2,000 to 20,000 range. MALDI-TOF MS yielded unique mass spectra for *C. vulgaris* depending on the age of the culture. For example, spectra from the late stationary phase of growth were different from other growth phases with many growth phase-specific peaks in the low mass range. Spectra from earlier growth phases shared peaks, such as m/z 2043, 2638, and 2659. Interestingly, spectra from late stationary phase cultures did not share these peaks and exhibited unique peaks at m/z 2033, 2862, and 3018. This report of growth phase-specific MALDI-TOF spectra of microalgae suggests that: 1) the performance of MS-based approaches to microalgae characterization may require standardization and 2) MALDI-TOF may be useful for the rapid characterization of microalgae growth phases and monitoring of culture health.

Introduction

Microalgae have received steadily increasing attention over the years as a subject of scientific study and industry. This is due to the ability of many microalgae species to be mass cultured and harvested for products including pharmaceuticals, nutraceuticals, and biofuels [1]. The varying growth rates among microalgae species that are affected by a plethora of external factors such as pH, temperature, light, and nutrient availability. These external factors need to be controlled in order to produce and maintain microalgae cultures [2]. The issue with many microalgae culturing systems or photobioreactors is that they are either open or semi-open to external factors that can negatively affect cultures or allow predators and contaminants to enter [2][3]. For example, the open configuration of algae ponds leaves cultures susceptible to invasion by zooplankton predators, like *Brachionus*, which can lead to decreased production or culture destruction. Furthermore, contamination by undesired microalgae species can lead to the invading species out-competing the original species for resources. Due to these limitations, these culture systems need to be monitored consistently for contaminants in order to ensure maximum productivity. The methods described in this work lay the groundwork for methods to be used for the near real-time health monitoring of microalgae cultures.

The current methods for microalgae culture monitoring include analysis of morphology by brightfield light or electron microscopy [4]. More in depth methods for culture analysis include molecular techniques such as pulsed-field gel electrophoresis (PFGE) [5], 18s rDNA analysis [6], multilocus sequence typing (MLST) [7], and repetitive sequence-based polymerase chain reaction (rep-PCR) [8]. These techniques

are highly effective for the detection of invading predators and microalgae species, but the techniques require large amounts of labor, time, and resources that are regarded as impractical for near real-time monitoring of microalgae culture systems [9]. Additionally, these techniques do not allow for determination of growth phase of microalgae cultures.

In the past, matrix-assisted desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been used as a tool to accurately describe bacterial organisms at the genus, species, and more recently, strain levels [10]. Beyond bacteria, MALDI-TOF MS has also been used to describe viruses [11], fungi [12-15], and microalgae [4][7][16][17][18]. The current studies on MALDI analysis studies of microalgae are fewer in number compared to the viruses, fungi, and bacteria. Previously, we have demonstrated that MALDI-TOF MS is a technique useful for the rapid and accurate characterization of a collection of microalgae species. Our study demonstrated that MALDI-TOF MS was highly efficient in characterizing 31 microalgae strains with a level of discrimination equal to, if not greater than, molecular techniques [18]. Additionally, MALDI was able to characterize simple artificial mixtures of microalgae showing that they could yield mixture-specific peaks.

Emami and colleagues [17] utilized an intact cell method combined with MALDI-TOF MS to obtain spectra for *Dunaliella* species, but noted that culture age did not have an effect on spectra. However, their work showed that MALDI-TOF MS of *Dunaliella* demonstrated higher levels of taxonomic resolution than internal transcribed spacer (ITS) sequence analyses. In contrast to the work of Emami, Nicolau and colleagues [4]

observed, using a protein extraction method, that culture age did have an effect on diatom cultures. Furthermore, the work of Wirth *et al* [19][20] demonstrated that downstream analysis optimization allowed users to differentiate between pathogenic and non-pathogenic strains of *Prototheca* and Von Bergen *et al* [21] demonstrated that MALDI-TOF MS was a useful in the characterization of five different pathogenic species of *Prototheca*. These studies suggest that MALDI-TOF MS holds not only promise for the rapid characterization of microalgae, but for the characterization of microalgae species based on culture age as well [22][23].

In order to expand on the ability of MALDI-TOF MS to characterize the growth curve of microalgae, we concentrated on a single model organism, *Chlorella vulgaris* UTEX 395. Our specific objectives in this study were to determine if MALDI-TOF MS can: 1) determine the growth phase of an organism at a given time, and 2) yield distinct phase-dependent spectra. The growth curve for *C.* vulgaris was measured daily using optical density. A common protein extraction sample preparation method was used daily in order to characterize *C. vulgaris*. Our results suggest that MALDI-TOF MS was able to: 1) observe time-dependent changes in microalgae that are characteristic of different growth phases, 2) yield characteristic spectral fingerprints for growth phases, and 3) observe phase-dependent peaks in multiple growth phases.

Materials and Methods:

Reagents

MALDI matrix, α-cyano-4-hydroxycinnaminic acid (CHCA), and trifluoroacetic (TFA) acid were purchased from ACROS (Fair Lawn, NJ, USA). Acetonitrile (ACN)

was purchased from Alfa Aesar (Ward Hill, MA, USA). MALDI calibrants (ACTH 1-17 (2,093.46 Da), ACTH 18-39 (2,464.19 Da), Insulin Oxidized B (3,494.65 Da), Insulin (5,730.61 Da), Cytochrome C (12,362.00 Da), and Apomyoglobin (16,952.30 Da)) and formic acid (FA) were purchased from Sigma (St. Louis, MO, USA). Ultrapure was filtered using a Milli-Q integral water purification system (Millipore Corporation, Billerica, MA, USA).

Microalgae Starter Cultures

The microalgae, *Chlorella vulgaris* UTEX 395, was provided by the Arizona Center for Algae Technology and Innovation (AzCATI; http://www.AzCATI.com). *Chlorella vulgaris* is a freshwater organism that was cultured by inoculating 75 mL of BG-11 [24] medium with 25 mL of microalgae at an optical density (750 nm) of 0.8 to produce a starting concentration of 0.2. The culture was maintained by sub-culturing every 6 days to an initial $O.D._{750}$ of 0.2 at day 0. Cultures were grown on an orbital shaker set at 120 rotations per minute under 75 $\mu mol/m^2/s$ of cool white fluorescent light at 20°C.

Characterization of Algae Growth Curve

Once starter cultures had reached an $O.D._{750}$ of 0.8 they were mixed together in a sterile flask and mixed thoroughly through shaking. Three 500 mL cultures in test tube-style semi-open photobioreactors were initialized by inoculating 375 mL of BG-11 medium with 125 mL of *C. vulgaris* culture. The cultures were grown under 75 $\mu mol/m^2/s$ of cool white fluorescent light at 20°C and aerated using house air at a flow rate of 2 container volumes per minute in order to facilitate mixing and gas exchange.

Carbon dioxide sparging was not used in our experimental set-up so that we could directly compare our work to previous work on MALDI and microalgae. The $O.D._{750}$ for all three *C. vulgaris* cultures was then measured every 24 hours for 12 consecutive days and the average O.D. was plotted in Sigma Plot in order to generate a growth curve. The 12 day cut-off was used because the cultures had entered stationary phase. Standard deviations for each time point were measured and plotted on the growth curve as well.

MALDI-TOF MS Sample Preparation

A previously described protein extraction method [25] was used as the base for the sample preparation method used in these experiments. A single milliliter of cells at an OD_{750} of 0.8 were washed with sterile milliQ-H_2O (mQ-H_2O) and then inactivated for 1 hour in a mixture of 300 µL mQ-H_2O and 900 µL of absolute ethanol. Cells were then centrifuged at 10,000 xg for two minutes at room temperature. The supernatant was then decanted and the sample was centrifuged once more at 10,000 xg and the supernatant decanted once more. The cells were then left to air dry for 1 minute. Following, 25 µL each of FA and ACN were then added to the algae pellet and vortexed vigorously. Cells were then centrifuged at 17,000 xg for five minutes at room temperature. The supernatant was collected immediately and used for MALDI analysis. The supernatant was then plated in triplicate 1 µL aliquots on a MSP 96 Polished Steel MALDI Target Plate (Bruker Daltonics, Billerica, MA, USA) and air-dried. The CHCA matrix solution was prepared by mixing 475 µL mQ-H_2O, 500 µL ACN, 25 µL 99.5% TFA, and 15 mg CHCA. Each 1 µL aliquot of supernatant was covered with 1 µL of CHCA matrix solution and allowed to air dry.

Data Acquisition

The Bruker Microflex LRF MALDI-TOF MS (Bruker Daltonics) was used for mass spectra acquisition. The MALDi was equipped with a 377 nm nitrogen laser controlled by Flex Control software (version 3.0; Bruker Daltonics). Mass spectra were collected automatically in the positive linear mode in the m/z 2,000 to 20,000 Da range. Ion source 1 was set at 20 kV, ion source 2 was set to 18.15 kV, and the lens was set to 9.05 kV. The laser frequency was set to 10 Hz. Mass spectrometer calibration was performed using a protein calibrant mixture using the proteins discussed above. Spectra for each technical replicate were generated from 500 laser shots collected in 100 shot bursts. In order for the spectra from the 100 shot burst to be included the following criteria had to be met: a base-peak signal-to-noise ratio (S:N) of 2 or greater, a peak width of 10 m/z, a minimum intensity threshold of 100, and a maximal number of peaks of 500. Three replicate MALDI mass spectra were obtained for each biological replicate at every sampling time. The Savitzky-Golay algorithm was used for peak smoothing. Following, the TopHat algorithm was used for baseline subtraction. Peak identification was facilitated by FlexAnalysis 3.0 software (Bruker Daltonics) and the information was transferred into a Microsoft Excel spreadsheet. From there, the average mass range and base peak signal-to-noise ratios were calculated in order to assess spectrum quality. In order for peaks to be considered different from one-another they had to vary by more than +/- 2 m/z.

Data Analysis

Analysis of *C. vulgaris* spectra was performed using BioNumerics software (v. 7; Applied Maths, Austin, TX, USA). Summary spectra for each time point were generated using the data from nine technical replicates representing three biological replicates. A total of 13 summary spectra were generated, 1 per time point. A similarity threshold of 65% was used to ensure that all technical replicate spectra were represented in the summarized spectra. Similarity was calculated using the Pearson correlation coefficient with 0% curve smoothing. The cluster analysis/dendrograms was generated using the the UPGMA algorithm. Lastly, a multi-dimensional scaling figure was generated based on the selected grouping for growth phases.

Results and Discussion

C. vulgaris Growth Curve and MALDI-TOF Spectra

The growth curve of *C. vulgaris* UTEX 395 was measured in triplicate using optical density (750nm) from day 0 until day 12. The lowest O.D. observed was 0.248 at day 0 and the O.D. maxed out at 1.265 on day 12. The logarithmic growth curve was then divided into phases based on the changes in optical density. The phases used here are early logarithmic phase containing day 0 and 1 (Fig 1A), late logarithmic phase containing day 2 and 3 (Fig 1b), early stationary phase containing days 4 and 5 (Fig 1c), middle stationary phase containing days 6, 7, and 8 (Fig 1d), and late stationary phase containing days 9, 10, 11, and 12 (Fig 1e).

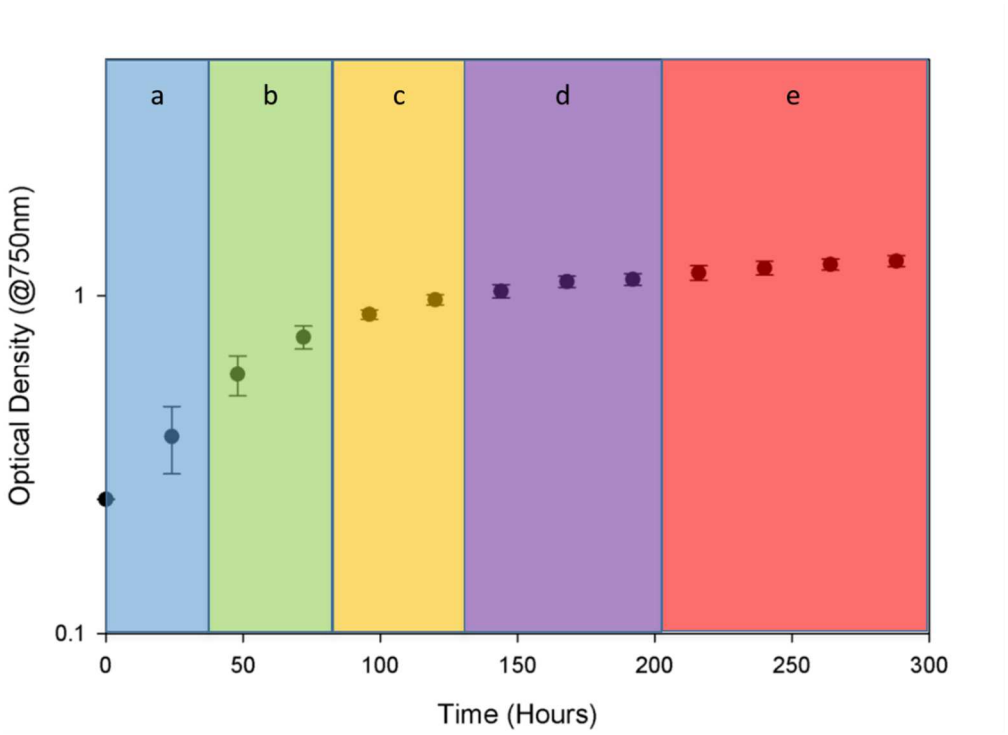

Figure 1. Growth Curve and Phase Separation for *C. vulgaris* UTEX 395 based on Average Optical Density.
Average optical densities with standard deviations for *C. vulgaris* at 24 hour intervals separated into growth phases; early logarithmic (a), late logarithmic (b), early stationary (c), middle stationary (d), and late stationary (e).

MALDI-TOF MS yielded similar spectra for *C. vulgaris* at each time point with slight, but detectable variations. We examined a mass range of m/z 2,000 – 20,000. This mass range was broader than those used in previous studies [16] that utilized a mass range of m/z 4,000 - 20,000. Our increased mass range contained peaks that were essential for the characterization of growth phases for *C. vulgaris*. The base peak at m/z 4868 was observed in all growth phases for *C. vulgaris* (Table 1). The late stationary phase of growth has spectra that are markedly distinct from all other phases (Fig 2). Many of the observable low mass peaks in the earlier phases become dwarfed by the proliferation of low mass peaks unique to the late stationary phase. This change in

spectra in the latest growth phase may be accounted for by ion suppression [26]. The low mass peaks off the late stationary phase were a highly repetitive series of three peaks. The series consisted of two lower intensity peaks approximately 129 daltons apart followed by a high intensity peak approximately 27 daltons higher in mass. Each of the high intensity peaks were approximately 285 daltons apart.

Table 1. Peaks observed in spectra of microalgae growth phases.

m/z	Early Log	Late Log	Early Stationary	Middle Stationary	Late Stationary
2033					U
2043	S	S	S	S	
2070	U				
2182	S	S	S	S	
2193	S	S	S	S	
2604					U
2638	S	S	S	S	
2659	S	S	S	S	
2862					U
3018					U
4630	S	S	S		
4868	S	S	S	S	S
4888		S	S	S	
6195	S	S	S		
11034				U	

S = peak shared amongst all growth phases
U = peak unique to a specific growth phase

The number of peaks and mass ranges varied between *C. vulgaris* spectra at different time points. The mass range for early logarithmic phase was m/z 2,043 to 14,771, which was broader than previously described [6]. The mass ranges for late logarithmic phase, early and middle stationary phases were narrower at m/z 2,000 to 13,669. The late stationary phase had a similar mass range to the early logarithmic phase (m/z 2,005 to 14,774). Numbers of peaks also varied depending on growth phase as well.

The early logarithmic (Fig 2a) and late stationary (Fig 2e) phases has the highest number of peaks with each having an average of 55 peaks. The early (Fig 2c) and middle stationary (Fig 2d) phases had slightly lower average peak numbers of 46. The late logarithmic phase (Fig 2b) had the lowest average peak number of 43.

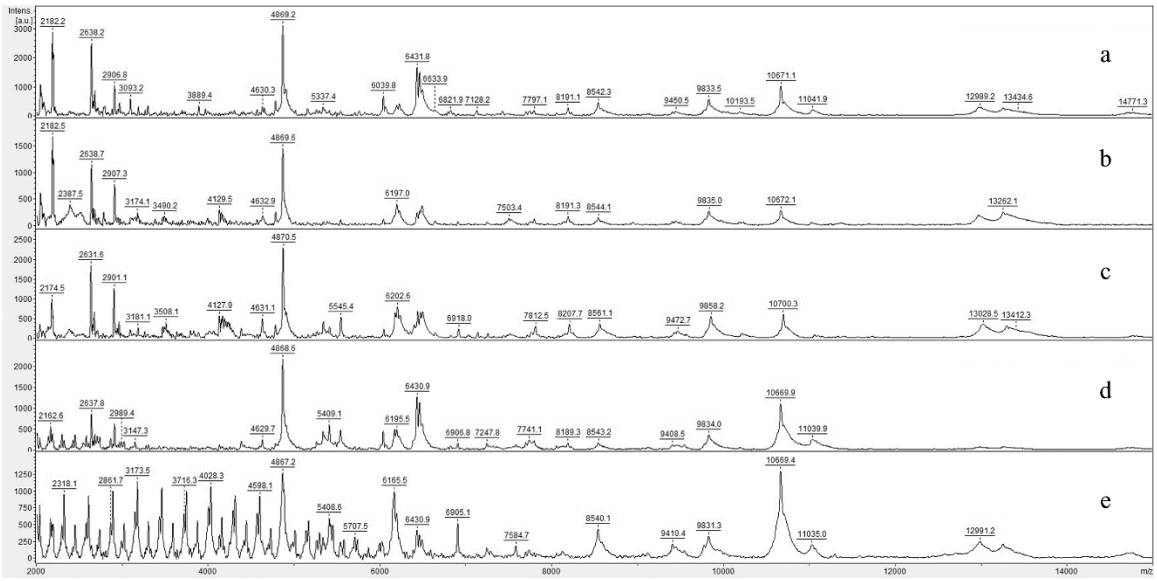

Figure 2. Representative MALDI-TOF spectra of *C. vulgaris* growth phases. Spectra representing the five proposed growth phases for *C. vulgaris* demonstrating the differences between phases; early logarithmic (a), late logarithmic (b), early stationary (c), middle stationary (d), and late stationary (e).

Our spectral data is similar to that of Nicolau and colleagues [4] in that both studies demonstrated high reproducibility amongst spectra, but also in that temporal changes resulted in changes in spectra as well. Their analysis of *Coscinodiscus* sp. demonstrated that as the culture grew from day 13 to day 30, the spectra shift from having multiple peaks within the m/z 2,000 to 4,000 to having a majority of peaks in the m/z 4,000 to 6,000 range. Additionally, their works also shows an increase in peak number. This is similar to our own work (Fig 2e) in that *C. vulgaris* saw an increase in

peak number once it entered the late stationary phase of growth. In contrast, the work of Emami and colleagues [17] demonstrated that there was not a detectable difference in spectral fingerprints based on culture age. The most noticeable difference between studies is that Emami and colleagues utilized an intact cell method while our study and the study by Nicolau utilized a protein extraction method for sample preparation. We suggest, based on this information, that in order to observe the effects of culture age on microalgae spectra, that the intracellular material must be examined rather than the cell wall and its composites.

MALDI-TOF Data

The differences in summarized growth phase spectra shown above were reflected in the cluster analysis of the *C. vulgaris* replicates (Fig 3a). The cluster analysis demonstrates a high level of clustering based on the proposed growth phases (Fig 3) with the late logarithmic phase demonstrating the lowest level of clustering which correlates with the grouping analysis shown below (Table 2). Grouping analyses of spectra also showed distinct growth phases matching those assigned above (Fig 3b; Table 2).

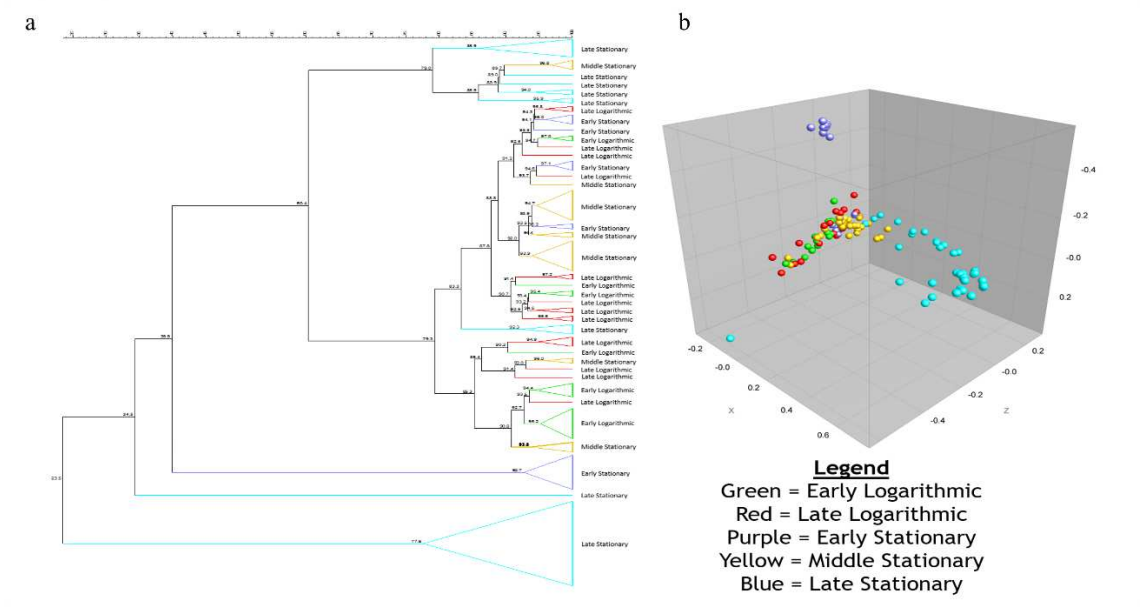

Figure 3. Similarity-based dendrogram (a) and multi-dimensional scaling figure (b) for *C. vulgaris* growth phases.
Spectra were clustered using the UPGMA algorithm and the MDS was generated using the metric algorithm.

While looking at the jackknife (Table 2) of the spectra grouped by growth phase we see that the early and late stationary phases group correctly 100% of the time. The early logarithmic phase demonstrated a high jackknife value (>94%) while grouping as the late logarithmic phase 5.6% of the time. The middle stationary phase also had a high jackknife value of 90.6% and grouped as the early logarithmic, early stationary, and late stationary phases 3.1% of the time each. The growth phase with the highest level of variability was the late logarithmic phase with a jackknife value of 72.2%. The late logarithmic phase was grouping as early stationary 16.7% of the time, the early logarithmic phase 5.6% of the time, and the middle stationary phase 5.6% of the time.

Table 2. Jackknife analysis of *C. vulgaris* growth phases.

	Early Log	Late Log	Early Stationary	Middle Stationary	Late Stationary
Early Log	94.4	5.6	0	3.1	0
Late Log	5.6	72.2	0	0	0
Early Stationary	0	16.7	100	3.1	0
Middle Stationary	0	5.6	0	90.6	0
Late Stationary	0	0	0	3.1	100

Conclusions

Our results suggest that MALDI-TOF MS is a viable method for characterizing the growth phases of microalgae cultures. To our knowledge, this is the first study to demonstrate that MALDI-TOF MS is capable of characterizing the growth phases of microalgae cultures. A previous study [17] on *Dunaliella* demonstrated that time-dependent changes in spectra were undetectable using intact cell methods. However, an additional recent study on diatom species [4] and culturing age, analyzed using a protein extraction method, showed that culture age played a significant role in spectral quality. Our study shows that a majority of the growth phases analyzed in this experiment had phase-specific peaks that could serve as biomarkers to indicate culture age. Appropriately, MALDI-TOF has the potential to act as a rapid and economical tool for monitoring microalgae cultures for changes such as phase shifts and contaminations. Our continued efforts include the development of an experimental protocol for analyzing zooplankton contaminants in microalgae cultures that would lead to rapid identification of predators.

Acknowledgements

This work was supported by the Arizona Center for Algae Technology and Innovation, the ASU New College of Interdisciplinary Arts & Sciences, and ASU Lightworks.

Author Contributions

Conceived and designed the experiments: DB TS TD. Performed the experiments: DB. Analyzed the data: DB TS TD. Contributed reagents/materials/analysis tools: TD. Wrote the paper: DB TS TD.

References

1. Ho SH, Ye X, Hasunuma T, Chang JS, Kondo A (2012) Perspectives on engineering strategies for improving biofuel production from microalgae–a critical review. Biotechnol Adv 32: 1448–1459.

2. Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S (2010) Biofuels from algae: challenges and potential. Biofuels 1: 763–784.

3. Smith VH, Crews T (2014) Applying ecological principles of crop cultivation in large-scale algal biomass production. Algal Res 4: 23–34.

4. Nicolau A, Sequeira L, Santos C, Mota M (2014) Matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) applied to diatom identification: influence of culturing age. Aquat Biol 20: 139–144.

5. Oh HS, Ah Kum K, Kim EC, Lee HJ, Choe KW, Oh MD (2008) Outbreak of Shewanella algae and *Shewanella putrefaciens* infections caused by a shared measuring cup in a general surgery unit in Korea. Infect Control Hosp Epidemiol 29: 742–748.

6. Lee HW, Roh SW, Cho K, Kim KN, Cha IT, Yim KJ, et al. (2015) Phylogenetic analysis of microalgae based on highly abundant proteins using mass spectrometry. Talanta 132: 630–634.

7. Wei L, Xin Y, Wang D, Jing X, Zhou Q, Su X, et al. (2013) Nannochloropsis plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. BMC Genomics 14: 534–550.

8. Godhe A, Anderson DM, Rehnstam-Holm AS (2002) PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. Harmful Algae 1: 375–382.

9. Giebel R, Worden C, Rust SM, Kleinheinz GT, Robbins M, et al. (2010) Microbial fingerprinting using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS): applications and challenges. Adv Appl Microbiol 71: 149–184.

10. Sandrin TR, Goldstein JE, Schumaker S (2013) MALDI-TOF MS profiling of bacteria at the strain level: a review. Mass Spectrom Rev 32: 188–217.

11. Calderaro A, Arcangeletti MC, Rodighiero I, Buttrini M, Gorrini C, Motta F, et al. (2014) Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry applied to virus identification. Sci Rep 4.

12. Pavlovic M, Mewes A, Maggipinto M, Schmidt W, Messelhausser U, Balsliemke J, et al. (2014) MALDI-TOF MS based identification of food-borne yeast isolates. J Microbiol Methods 106: 123–128.

13. Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M (2013) Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF mass spectrometry. Med Mycol 51: 580–585.

14. Wieme AD, Spitaels F, Vandamme P, et al. (2014) Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a monitoring tool for in-house brewer's yeast contamination: a proof of concept. J Inst Brew 120: 438–443.

15. Deak E, Charlton CL, Bobenchik AM, Miller SA, Pollett S, McHardy IH, et al. (2015) Comparison of the Vitek MS and Bruker Microflex LT MALDI-TOF MS platforms for routine identification of commonly isolated bacteria and yeast in the clinical microbiology laboratory. Diagn Micr Infec Dis 81: 27–33.

16. Andrade LM, Mendes MA, Kowalski P, Nascimento CAO (2015) Comparative study of different matrix/solvent systems for the analysis of crude lyophilized microalgal preparations using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 29: 295–303.

17. Emami K, Hack E, Nelson A, Brain CM, Lyne FM, Mesbahi E, et al. (2015) Proteomic-based biotyping reveals hidden diversity within a microalgae culture collection: an example using *Dunaliella*. Sci Rep 5: 10036.

18. Barbano D, Diaz R, Zhang L, Sandrin T, Gerken H, Dempster T (2015) Rapid Characterization of Microalgae and Microalgae Mixtures Using Matrix-Assisted Laser

Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). PLoS ONE 10(8): e0135337.

19. Wirth H, von Bergen M, Murugaiyan J, Roesler U, Stokowy T, Binder H (2012) MALDI-typing of infectious algae of the genus Prototheca using SOM potraits. J Microbiol Methods 88: 83–97.

20. Murugaiyan J, Ahrholdt J, Kowbel V, Roesler U (2012) Establishment of a matrix-assisted laser desorption ionization time-of-flight mass spectrometry database for rapid identification of pathogenic achlorophyllous green micro-algae of the genus *Prototheca*. Clin Microbiol Infec 18: 461–467.

21. von Bergen M, Eidner MA, Schmidt F, Murugaiyan J,Wirth H, Binder H, et al. (2009) Identification of harmless and pathogenic algae of the genus Prototheca by MALDI-MS. Proteomics Clin Appl 3: 774–784.

22. Smith VH (2014). Advances in algal biofuels and bioproducts. Industrial Biotechnology 10: 159–161.

23. Knoshaug EP, Darzins A (2011). Algal Biofuels: The Process. Chem Engin Prog 107: 37–47.

24. Stainer RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (Order Chroococcales). Bacteriol Rev 35: 171–205.

25. Freiwald A, Sauer S (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc 4: 732–742.

26. Holland RD, Wilkes JG, Cooper WM, Alusta P, Williams A, Pearce B, et al. (2014) Thymol treatment of bacteria prior to matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis aids in identifying certain bacteria at the subspecies level. Rapid Commun Mass Spectrom 28: 2617–2626.

Summary Conclusion and Future Work

The purpose of this thesis was to evaluate MALDI-TOF MS as a method for rapid and accurate characterization of economically-relevant microalgae. This work was completed in two research chapters that emphasized the characterization of a microalgae collection, simple mixture analysis, and growth curve characterization. The results generated were compared to those found in the current literature. Lastly, this thesis presents those questions that require further investigation in order to further evaluate MALDI-TOF MS as a near-real time microalgae monitor.

Chapter three focused on the efficacy of MALDI-TOF MS as a tool for the rapid and accurate characterization of a collection of microalgae vital to industry. They key points from this chapter are:

- MALDI-TOF MS represents an effective alternative to conventional techniques for the characterization of microalgae.
- MALDI-TOF MS is just as effective, if not more so, as molecular methods for the identification of microalgae.
- MALDI-TOF MS is a potential tool for strain-level differentiation of microalgae.
- MALDI-TOF MS can be used to analyze simple mixtures leading to identification of contaminants or predators.

Chapter four focused on the ability of MALDI-TOF MS to detect changes in spectra based on microalgae culture age. The key points from this chapter are:

- MALDI-TOF MS is capable of detecting changes in algae culture over time.
- MALDI-TOF MS is capable of detecting changes in microalgae spectra over time.

- MALDI-TOF MS can be used to detect growth phase specific peaks.

This study should be further expanded to analyze microalgae cultures that have been intentionally contaminated with an invading microalgae species and/or a zooplankton predator. In order to proceed with this work, the following recommendations should be regarded to increase the likelihood of success.

- Careful selection of specific marine and freshwater microalgae to be used as model organisms. Organisms should be susceptible to damage and/or culture failure when preyed upon by predators or competing with other microalgae.
- Careful selection of microalgae to be used as an invading organism capable of out-competing the original organism.
- Careful selection of zooplankton predators capable of large amounts of grazing and the potential for causing catastrophic culture crashes. Currently, *Brachionus plicatilis* and *B. calyciflorus* are being examined as model predatory organisms.
- Planning for an ideal culturing set-up to maximize microalgae growth and allows proliferation of zooplankton predators. The culture conditions should be optimized to support the growth of both the predator and the microalgae. This could include pH monitoring and control, nitrogen and phosphorous monitoring, and monitoring culture agitation as it may be too turbulent for predator proliferation.
- Optimization of MALDI-TOF MS protocols to further improve data collection. The common method used in the previously described studies is effective for analyzing *C. vulgaris*, however, laboratory experiments indicates that the method

requires revision for the microalgae *Nannochloropsis oceanica*. This difficulty may be due to the complexity of the cell wall of a marine organism and may require a harsher method for lysing the cell.

- Evaluate the use of carbon dioxide sparging and its effect on microalgae spectra. Previous studies by Emami, Nicolau, Wei, and Lee did not utilize carbon dioxide sparging. The use of carbon dioxide may have an effect on MALDI spectra and that effect should be analyzed for optimized microalgae characterization.

Works Cited

1. Pulz O, Scheibenbogen K (1998) Photobioreactors: Design and Performance with Respect to Light Energy Input. Bioprocess and Algae Reactor Technology, Apoptosis. pp. 123–152

2. Singh A, Nigam PS, Murphy JD (2010) Mechanism and challenges in commercialisation of algal biofuels. Bior Tech 102: 26-34.

3. Pavlovic M, Mewes A, Maggipinto M, Schmidt W, Messelhausser U, Balsliemke J, et al. (2014) MALDI-TOF MS based identification of food-borne yeast isolates. J Microbiol Methods 106: 123–128.

4. Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M (2013) Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF mass spectrometry. Med Mycol 51: 580–585.

5. Wieme AD, Spitaels F, Vandamme P, et al. (2014) Application of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry as a monitoring tool for in-house brewer's yeast contamination: a proof of concept. J Inst Brew 120: 438–443.

6. Deak E, Charlton CL, Bobenchik AM, Miller SA, Pollett S, McHardy IH, et al. (2015) Comparison of the Vitek MS and Bruker Microflex LT MALDI-TOF MS platforms for routine identification of commonly isolated bacteria and yeast in the clinical microbiology laboratory. Diagn Micr Infec Dis 81: 27–33.

7. Sandrin TR, Goldstein JE, Schumaker S (2013) MALDI-TOF MS profiling of bacteria at the strain level: a review. Mass Spectrom Rev 32: 188–217.

8. Barbano D, Diaz R, Zhang L, Sandrin T, Gerken H, Dempster T (2015) Rapid Characterization of Microalgae and Microalgae Mixtures Using Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS). PLoS ONE 10(8): e0135337.

9. Karas M, Bachmann D, Bahr U, and Hillenkamp F. 1987. Matrix-Assisted Ultraviolet Laser Desorption of Non-Volatile Compounds. Int J Mass Spectrom Ion Processes 78: 53-68.

10. Cain TC, Lubman DM, Weber Jr WJ. 1991. Differentiation of bacteria using protein profiles from matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 8: 1026-1030.

11. Dieckmann R, Helmuth R, Erhard M, Malorny B. 2008. Rapid classification and identification of *Salmonellae* at the species and subspecies levels by whole-cell matrix-

assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 74: 7767-7778.

12. Benagli C, Rossi V, Dolina M, Tonolla M, Petrini O. 2011. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for the identification of clinically relevant bacteria. PLoS ONE 6:e16424

13. Siegrist TJ, Anderson PD, Huen WH, Kleinheinz GT, McDermott CM, Sandrin TR. 2007. Discrimination and characterization of environmental strains of *Escherichia coli* by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). J Microbiol Methods 68: 554-562.

14. Giebel RA, Fredenberg W, Dandrin TR. 2008. Characterization of environmental isolates of *Enterococcus* spp. by matrix-assisted laser desorption/ionization mass spectrometry. Water Res 42: 931-940.

15. Karas M, Kruger R. 2003. Ion formation in MALDI: the cluster ionization mechanism. Chem Rev 103: 427-440.

16. Erhard M, von Dohren H, Jungblut P. 1997. Rapid typing and elucidation of new secondary metabolites of intact cyanobacteria using MALDI-TOF mass spectrometry. Nat Biotechnol 15: 906-909.

17. Lundquist M, Caspersen MB, Wikstrom P, Forsman M. 2005. Discrimination of *Francisella tularensis* subspecies using surface enhanced laser desorption ionization mass spectrometry and multivariate data analysis. FEMS Microbiol Lett 243: 303-310.

18. Lee HW, Roh SW, Cho K, Kim KN, Cha IT, Yim KJ, Song HS, Nam YD, Oda T, Chung YH, Kim SJ, Choi JS, Kim D. 2015. Phylogenetic Analysis of Microalgae Based on Highly Abundant Proteins using Mass Spectrometry. Talanta 132: 630-634.

19. Godhe A, Anderson DM, Rehnstam-Holm AS (2002) PCR amplification of microalgal DNA for sequencing and species identification: studies on fixatives and algal growth stages. Harmful Algae 1: 375-382.

20. Oh HS, Ah Kum K, Kim EC, Lee HJ, Choe KW, Oh MD (2008) Outbreak of Shewanella algae and *Shewanella putrefaciens* infections caused by a shared measuring cup in a general surgery unit in Korea. Infect Control Hosp Epidemiol 29: 742-748.

21. Wei L, Xin Y, Wang D, Jing X, Zhou Q, Su X, et al. (2013) Nannochloropsis plastid and mitochondrial phylogenomes reveal organelle diversification mechanism and intragenus phylotyping strategy in microalgae. BMC Genomics 14: 534-550.

22. Hillenkamp F, Karas M. 1991. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Biopolymers. Anal Chem 63: 1193-1202.

23. Posthumus M, Kistemaker P, Meuzelaar H, Ten Noever de Brauw M. 1978. Laser Desorption-Mass Spectrometry of Polar Nonvolatile Bio-Organic Molecules. Anal Chem 50: 985-991.

24. Karas M, Bachmann D, Hillenkamp F. 1985. Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules. Anal Chem 57: 2935-2939.

25. Karas M, Bachmann D, Bahr U, Hillenkamp F. 1987. Matrix-assisted ultraviolet laser desorption of non-volatile compounds. Int J Mass Spectrom Ion Processes 78: 53-68.

26. Karas M, Ute B, Strupat K, Hillenkamp F. 1995. Matrix Dependence of Metastable Fragmentation of Glycoproteins in MALDI TOF Mass Spectrometry. Anal Chem 67: 675-679.

27. Karas M, Hillenkamp F. 1988. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10000 Daltons. Anal Chem 60: 2301-2303.

28. Zhao S, Somayajula K, Sharkey A, Hercules D. 1991. Novel Method for Matrix-Assisted Laser Mass Spectrometry of Proteins. Anal Chem 63: 450-453.

29. Hillenkamp F, Karas M. 1991. Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry of Biopolymers. Anal Chem 63: 1193-1202.

30. Fagerquist CK, Sultan O. 2011. Induction and identification of disulfide-intact and disulfide-reduced beta-subunit of shiga toxin 2 from *Escherichia coli* O157:H7 using MALDI-TOF MS/MS and top-down proteomics. Analyst 136: 1739.

31. Mortz E. 1996. Sequence tag identification of intact proteins by matching tandem mass spectral data against sequence databases. Proc Natl Acad Sci USA 93: 8264-8267.

32. Fox K, Fox A, Rose J, Walla M. 2011. Speciation of coagulase negative staphylococci, isolated from indoor air, using SDS PAGE gel bands of expressed proteins followed by MALDI TOF MS and MALDI TOF-TOF MS-MS analysis of tryptic peptides. J Microbiol Methods 84: 243-250.

33. Karas M, Hillenkamp F. 1993. Matrix-Assisted Laser Desorption Ionization Mass Spectrometry – Fundamentals and Applications. AIP Conference Proceedings. 288: 447-458.

34. Bahr U, Karas M, Hillenkamp F. 1994. Analysis of Biopolymers by Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry. Anal Chem 348: 783-791.

35. Pavlovic M, Mewes A, Maggipinto M, Schmidt W, Messelhausser U, Balsliemke J, et al. 2014. MALDI-TOF MS Based Identification of Food-Borne Yeast Isolates. J Microbiol Methods 106: 123-128.

36. Packeu A, Hendrickx M, Beguin H, Martiny D, Vandenberg O, Detandt M. 2013. Identification of the *Trichophyton mentagrophytes* complex species using MALDI-TOF Mass Spectrometry. Med Mycol 51: 580-585.

37. Wieme AD, Spitaels F, Vandamme P, et al. 2014. Application of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry as a Monitoring Tool for In-House Brewer's Yeast Contamination: a Proof of Concept. J Inst Brew 120: 438-443.

38. Deak E, Charlton CL, Bobechik AM, Miller SA, Pollett S, McHardy IH, et al. 2015. Comparison of the Vitek MS and Bruker Microflex LT MALDI-TOF MS Platforms for Routine Identification of Commonly Isolated Bacteria and Yeast in the Clinical Microbiology Laboratory. Diagn Micr Infec Dis 81: 27-33.

39. Jarman KH, Cebula ST, Saenz AJ, Petersen CE, Valentine NB, Kingsley MT, Wahl KL. 2000. An Algoriithm for Automated Bacterial Identification Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry. Anal Chem 72: 1217-1223.

40. Hsieh SY, Tseng CL, Lee YS, Kuo AJ, Sun CF, Lin YH, Chen JK. 2007. Highly Efficient Classification and Identification of Human Pathogenic Bacteria by MALDI-TOF MS. Mol Cell Proteomics 7: 448-456.

41. Nicolau A, Sequeira L, Santos C, Mota M. 2014. Matrix-Assisted Laser Desorption/Ionisation Time-of-Flight Mass Spectrometry (MALDI-TOF MS) Applied to Diatom Identification: Influence of Culturing Age. Aquat Biol 20: 139-144.

42. Barbano DL, Diaz R, Zhang L, Sandrin T, Gerken H, Dempster T. 2015. Rapid Characterization of Microalgae and Microalgae Mixtures Using Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). PLoS ONE 10: e0135337.

43. Andrade LM, Mendes MA, Kowalski P, Nascimento CAO. 2015. Comparative Study of Different Matrix/Solvent Systems for the Analysis of Crude Lyophilized Microalgal Preparations using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Rapid Commun Mass Spectrom 29: 295-303.

44. Wieser A, Schneider L, Jung J. 2012. MALDI-TOF MS in Microbiological Diagnostics – Identification of Microorganisms and Beyond (Mini Review). Appl Microbiol Biotechnol 93: 965-974.

45. Wahl KL, Wunschel SC, Jarman KH, Valentine NB, Petersen CE, Kingsley MT, Zartolas KA, Saenz AJ. 2002. Analysis of Microbial Mixtures by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Anal Chem 74: 6191-6199.

46. Mahe P, Arsac M, Chatellier S, Monnin V, Perrot N, Mailler S, Girard V, Ramjeet M, Surre J, Lacroix B, vam Belkum A, Veyrieras JB. 2014. Automatic Identification of Mixed Bacterial Species Fingerprints in a MALDI-TOF Mass-Spectrum. Bioinformatics 30: 1280-1286.

47. Stackebrandt E, Pauker O, Erhard M. 2005. Grouping *Myxococci* (*Corallococcus*) strains by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry: Comparison with gene sequence phylogenies. Curr Microbiol 50: 71-77.

48. Moura H, Woolfitt AR, Carvalho MG, Pavlopoulos A, Teixeira LM, Satten GA, Barr JR. 2008. MALDI-TOF mass spectrometry as a tool for differentiation of invasive and noninvasive *Streptococcus pyogenes* isolates. FEMS Immunol Med Microbiol 53: 333-342.

49. Schumaker S, Borror CM, Sandrin TR. 2012. Automating data acquisition affects mass spectrum quality and reproducibility during bacterial profiling using an intact cell sample preparation method with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 26: 243-253.

50. Vargha M, Takats Z, Konopka A, Nakatsu CH. 2006. Optimization of MALDI-TOF MS for strain level differentiation of *Arthrobacter* isolates. J Microbiol Methods 66: 399-409.

51. Schmidt F, Fiege T, Hustoft HK, Kneist S, Thiede B. 2009. Shotgun mass mapping of *Lactobacillus* species and subspecies from caries related isolates by MALDI-MS. Proteomics 9: 1994-2003.

52. Fujinami Y, Kikkawa HS, Kurosaki Y, Sakurada K, Yoshino M, Yasuda J. 2011. Rapid discrimination of *Legionella* by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. Microbiol Res 166: 77-86.

53. Sedo O, Sedlacek I, Zdrahal Z. 2011. Sample preparation methods for MALDI-MS profiling of bacteria. Mass Spectrom Rev 30: 417-434.

54. Liu H, Du Z, Wang J, Yang R. 2007. Universal sample preparation method for characterization of bacteria by matrix-assisted laser desorption ionization-time of flight mass spectrometry. Appl Environ Microbiol 73: 1899-1907.

55. Freiwald A, Sauer S (2009) Phylogenetic classification and identification of bacteria by mass spectrometry. Nat Protoc 4: 732-742.

56. Demirev PA, Fenselau C. 2008. Mass spectrometry for rapid characterization of microorganisms. Annu Rev Anal Chem 1: 71-93.

57. Sandrin TR, Goldstein JE, Schumaker S. 2013. MALDI TOF MS Profiling of Bacteria at the Strain Level: A Review. Mass Spectrom Rev 32: 188-217.

58. Holland RD, Wilkes JG, Rafii F, Sutherland JB, Persons CC, Voorhees KJ, Lay JO, Jr. 1996. Rapid identification of intact whole bacteria based on spectral patterns using matrix-assisted laser desorption/ionization with time-of-flight mass spectrometry. Rapid Commun Mass Spectrom 10: 1227-1232.

59. Eng JK, McCormack AL, Yates JR III. 1994. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 5:976-989.

60. Fenselau C, Russell S, Swatkoski S, Edwards N. 2007. Proteomic strategies for rapid characterization of micro-organisms. Eur J Mass Spectrom 13: 35.

61. Dai Y, Li L, Roser DC, Long SR. 1999. Detection and identification of low-mass peptides and proteins from solvent suspensions of *Escherichia coli* by high performance liquid chromatography fractionation and matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Spectrom 13: 73-78.

62. Demirev PA, Ho YP, Ryzhov V, Fenselau C. 1999. Microorganism identification by mass spectrometry and protein database searches. Anal Chem 71: 2732-2738.

63. Teramoto K, Kitagawa W, Sato H, Torimura M, Tamura T, Tao H. 2009. Phylogenetic analysis of *Rhodococcus erythropolis* based on the variation of ribosomal proteins as observed by matrix-assisted laser desorption ionization-mass spectrometry without using genome information. J Biosci Bioeng 108: 348-353.

64. Sauer S, Freiwald A, Maier T, Kube M, Reinhardt R, Kostrzewa M, Geider K. 2008. Classification and identification of bacteria by mass spectrometry and computational analysis. PLoS ONE: e2843.

65. Lartigue, MF, Hery-Arnaud G, Haguenoer E, Domelier AS, Schmit P-O, van der Mee-Marquet N, Lanotte P, Mereghetti L, Kostrzewa M, Quentin R. 2009. Identification

of *Streptococcus agalactiae* isolates from various phylogenetic lineages by matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 47: 2284-2287.

66. Stephan R, Cernela N, Ziegler D, Pfluger V, Tonolla M, Ravasi D, Fredriksson-Ahomaa M, Hachler H. 2011. Rapid species specific identification and subtyping of *Yersinia enterocolitica* by MALDI-TOF mass spectrometry. J Microbiol Methods 87: 150-153.

67. Hanh D, Mirza B, Benagli C, Vogel G, Tonolla M. 2011. Typing of nitrogen-fixing *Frankia* strains by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry. Syst Appl Microbiol 34: 63-68.

68. Rupf S, Breitung K, Schellenberger W, Merte K, Kneist S, Eschrich K. 2005. Differentiation of mutans streptococci by intact cell matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Oral Microbiol Immunol 20: 267-273.

69. Pierce CY, Barr JR, Woolfitt AR, Moura H, Shaw EI, Thompson HA, Massung RF, Fernandez FM. 2007. Strain and phase identification of the U.S. category B agent *Coxiella burnetii* by matrix assisted laser desorption/ionization time-of-flight mass spectrometry and multivariate pattern recognition. Anal Chim Acta 583: 23-31.

70. Chen P, Lu Y, Harrington PB. 2008. Biomarker profiling and reproducibility study of MALDI-MS measurements of *Escherichia coli* by analysis of variance-principal component analysis. Anal Chem 80:1474-1481.

71. Lee RE. Phycology. 3rd ed. Cambridge: Cambridge University Press; 1999.

72. Benemann JR. Microalgae Biotechnology: Products, Processes and Opportunities Volume 1. 1st ed. Washington: Omec International, Inc; 1985.

73. Rittmann BE. 2008. Opportunities for renewable bioenergy using microorganisms. Biotechnol Bioeng 100: 203-212.

74. van den Hoek C, Mann DG, Jahns HM. Algae: An Introduction to Phycology. 1st ed. Cambridge: Cambridge University Press; 1995.

75. Villareal TA, Joseph L, Brzezunski MA, Shipe RF, Lipschultz F, Altabet MA. 1999. Biological and chemical characteristics of the giant diatom *Ethmodiscus* (Bacillariophyceae) in the central north pacific gyre. J Phycol 35: 896-902.

76. Aaronson S. 1974. The biology and ultrastructure of phagotrophy in *Ochromonas Danica* (Chrysophyceae: Chrysomonadida). J Gen Microbiol 83: 21-29.

77. Nicholls KH. 1988. Descriptions of three new species of Mallomonas (Chrysophyceae): M. hexagonis, M. liturata and M. galeiformis. Br Phycol J 23: 159-166.

78. Bartley ML, Wiebke JB, Corcoran AA, Holguin, FO, Schaub T. 2013. Effects of salinity on growth and lipid accumulation of biofuel microalga *Nannochloropsis salina* and invading organisms. Biom Bioe 54: 83-88.

79. Krienitz L, Wirth M. 2006. The high content of polyunsaturated fatty acids in *Nannochloropsis limnetica* (Eustigmatophyceae) and its implication for food web interactions, freshwater aquaculture and biotechnology. Limnologica 36: 204-210.

80. Brune D, Lundquist T, Benemann J. 2009. Microalgal biomass for greenhouse gas reductions: potential for replacement of fossil fuels and animal feeds. J Environ Eng 135: 1136-1144.

81. Benemann JR. Microalgae Biotechnology: Products, Processes and Opportunities Volume 2. 1st ed. Washington: Omec International, Inc; 1987.

82. Sharif Hossain ABM, Salleh A (2008) Biodiesel fuel production from algae as renewable energy. Am J Biochem Biotechnol 4: 250-254.

83. Hook M, Xu T (2013) Depletion of fossil fuels and anthropogenic climate change – a review. Energy Policy 52: 797-809.

84. Hannon M, Gimpel J, Tran M, Rasala B, Mayfield S. 2010. Biofuels from algae: challenges and potential. Biofuels 1: 763-784.

85. Environmental Protection Agency. Climate Change: Greenhouse Gas Emissions [Internet]. [Cited 2016 April 17] Available from: https://www3.epa.gov/climatechange/ghgemissions/

86. Chisti, Y. 2007. Biodiesel from Microalgae. Biotech Adv 25: 294-306.

87. Food and Agriculture Organization of the United Nations. Algal Production [Internet]. [Cited 2016 March 16] Available from: http://guides.is.uwa.edu.au/c.php?g=324981&p=2178452

88. Guillard RRL. Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH, editors. Culture of marine invertebrate animals. New York: Plenum Press; 1975. pp. 29-60.

89. Stainer RY, Kunisawa R, Mandel M, Cohen-Bazire G (1971) Purification and properties of unicellular blue-green algae (Order Chroococcales). Bacteriol Rev 35: 171-205

90. Tredici MR. 2004. Mass Production of Microalgae: Photobioreactors. In: Richmond A (Ed.), Handbook of Microalgal Culture: Biotechnology and Applied Phycology. Blackwell Publishing, Iowa, USA, pp. 178-214.

91. Oswald WJ. 1988. Micro-algae and waste-water treatment. In: Borowitzka MA, Borowitzka LJ (Eds.), Microalgal biotechnology. Cambridge University Press, New York, USA, pp. 305-328.

92. Borowitzka MA. 2005. Culturing microalgae in outdoor ponds. In: Andersen, IRA (Ed.), Algal Culturing Techniques. Elsevier, Academic Press, New York, USA, pp. 205-218.

93. Kumar K, Mishra SK, Shrivastav A, Park MS, Yang J-W. 2015. Recent trends in the mass cultivation of algae in raceway ponds. Renew Sust Energ Rev 51: 875-885.
84. Borowitzka MA, Moheimani NR. 2013. Open Pond Culture Systems. J Appl Phycol 5: 133-152.

95. Mata TM, Martins AA, Caetano NS. 2010. Microalgae for Biodiesal Production and Other Applications: A Review. Renew Sust Energ Rev 14: 217-232.

96. Lee YK. 2001. Microalgal Mass Culture Systems and Methods: Their Limitation and Potential. J Appl Phycol 13: 307-315.

APPENDIX A

Co-Author Manuscript Permissions

All co-authors for manuscripts have been informed on the use of manuscripts in the documentation. Permission to utilize the manuscripts was obtained from all co-authors individually.