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CHLOROPHYLLIDE *A*: FACT OR ARTIFACT - RESOLUTION OF THE CHLOROPHYLLIDE *A* PROBLEM IN THE ROUTINE MEASUREMENT OF PLANKTONIC CHLOROPHYLL *A*

A Thesis

Presented to the

Faculty of

Moss Landing Marine Laboratories

California State University Monterey Bay

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Marine Science

by

Sierra Claire Helmann

Summer 2019

CALIFORNIA STATE UNIVERSITY MONTEREY BAY

The Undersigned Faculty Committee Approves the

Thesis of Sierra Claire Helmann

**CHLOROPHYLLIDE *A*: FACT OR ARTIFACT -
RESOLUTION OF THE CHLOROPHYLLIDE A PROBLEM IN THE ROUTINE
MEASUREMENT OF PLANKTONIC CHLOROPHYLL *A***

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by

Sierra Helmann

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DEDICATION

This thesis is dedicated to my students in Student Oceanography Club.

“Life is not easy for any of us. But what of that ? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing must be attained.” – Marie Curie

ABSTRACT

Chlorophyllide *a*: Fact or Artifact -
Resolution of the Chlorophyllide *a* Problem in the Routine Measurement of planktonic Chlorophyll *a*

by

Sierra Claire Helmann
Masters of Science in Marine Science
California State University Monterey Bay, 2019

Chlorophyll *a* serves as the routine proxy for most estimates of phytoplankton biomass in limnology and oceanography. It is well known that enzymatic chlorophyllase activity breaks chlorophyll *a* into the degradation product chlorophyllide *a* (chlide *a*). This degradation could result in potentially large underestimates of the true chlorophyll *a* concentration when the analysis is made by modern chromatographic methods. The goal of this project was to determine proper protocol in the methodological elimination of artifactual chlorophyllide *a* formed during extraction of chlorophyll pigments through the application of a microwave-assisted solvent extraction technique. This study aimed to answer the question: Is chlorophyllide *a* a fact or artifact? Previous literature has suggested that chromatographically analyzed chlorophyllide *a* might be either an artifact of the extraction process or an in situ indicator of senescent, physiologically compromised phytoplankton due to environmental stressors. This study addresses this decades-old problem for chlorophyll analysis. The microwave technique described inhibits chlorophyllase enzymatic activity, preventing the artifactual production of chlorophyllide *a*. The heat of the microwave technique not only denatures the chlorophllyase enzyme activity, but also evaporates filter-retained water (known to promote enzyme activity in solvents such as acetone and methanol). This technique results in a sizeable increase in the yield of extracted chlorophyll *a* that was up to four times greater than the routine protocol of cold temperature solvent-soak technique using 90% acetone. Microwave-assisted extraction methodology consistently yielded higher concentrations of total chlorophyll *a*-like pigments than commonly employed solvent-soak technique used in long term sampling programs such as JGOFS, HOTS and BATS. The result of this study provides a method that 1) eliminates artifactual chlorophyllide *a* production and 2) increases the extraction yield of photosynthetic pigments in phytoplankton, including both chlorophylls and carotenoids. This new technique may have significant implications for estimates of oceanic primary production.

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Thank you for the support from my family, friends and professors.

Introduction

Chlorophyll *a* molecules are fundamental to the biological process of photosynthesis, both as the primary reaction center pigments (P680 and P700) and as accessory light absorbers (antenna pigments) (Allen and Williams 1998). As a result, chlorophyll *a* is one of the most often measured analytical parameters in biological oceanography research programs (Wernand et al. 2013). Chlorophylls are tetrapyrroles, cyclic compounds made up of 4 fused pyrrole rings. Pyrroles are 5 member rings with four carbons and one nitrogen atom. In chlorophyll *a*, a Mg ion is in the center of the four pyrrole rings. The most abundant tetrapyrroles are chlorophylls *a*, *b* and *c*. Chlorophylls *a* and *b* are both magnesium-containing chlorins and have long hydrophobic phytol chains that make up their structure; chlorophyll *c* (c_1, c_2 and c_3), is actually a magnesium-containing porphyrin with the phytol chain absent. The structure of chlorophyll *a* can be modified by the removal of the phytol chain catalyzed by the enzyme chlorophyllase, which generates chlorophyllide *a*. Chlorophyllide *a* is also an intermediate in chlorophyll biosynthesis (Fig. 1).

There are several different forms of chlorophyll that function as antenna absorbance pigments for a variety of organisms. The five major types of chlorophylls are chlorophylls *a*, *b*, *c*, *d* and bacteriochlorophyll; more recent studies have identified chlorophylls *e* and *f*, but not in widespread distribution (Airs et al. 2014). Chlorophyll *a* plays an essential role in oxygenic photosynthesis because it serves as the primary reaction center (P700 or P680) leading to electron transport. All of the other chlorophylls (and most carotenoids) are considered accessory pigments (Table 1).

Table 1: Accessory pigments, their maximal absorbance wavelengths, and associated organisms. (adapted from Roy et al. 2011).

Types of Chlorophylls	Wavelengths Absorbed	Comments
Chlorophyll *a* allomer		
Chlorophyll *a* epimer	Same as Chlorophyll *a* 432,616, 664 nm	Occurs in all photosynthetic algae and plants
Chlorophyll b epimer,	Same as Chlorophyll *a* 432,616, 664 nm	Occurs in slightly acidic or basic extracts using polar solvents
Chlorophyll c1	In acetone: 446,578,629 nm	Minor pigment of some red algae; major pigment in planktonic Chromophytes.
Chlorophyll c2	In acetone: 450, 581, 629 nm	Minor pigment of some red algae; major pigment in planktonic Chromophytes.
Chlorophyll c2-monogalactosyldiacyl glyceride ester	In acetone; 453, 582, 631 nm	Found as the dominant pigment in some Haptophytes
Chlorophyll c3	In acetone: 452, 586, 626 nm	Bolidophytes, many diatoms,haptophytes and some dinoflagellates. Sometimes found in dictychophytes and pelagophytes
Chlorophyllide *a*	**In acetone: 412,431,580, 617,664 nm**	**An alteration product of chlorophyll a, occurs in senescent algae, damaged diatoms and zooplankton fecal pellets. Extraction artefact for algae with highly active chlorophyllase enzyme**
Chlorophyllide b	In acetone: 458,596,646 nm	Occurs in senescent algae, zooplankton fecal pellets
Divinyl chlorophyll a	In acetone: 436,~615,661 nm	Found in *Prochlorococcus* sp.
Divinyl chlorophyll b	In acetone: 460,595,644 nm	Found in *Prochlorococcus* sp.
Magnesium 2,4-	In acetone:	Found in prasinoxanthin-containing algae,

divinylpheophorphyrin a5 monomethyl ester	438,575,625 nm	trace amounts in *Euglena*, unidentified chlorarachniophyte, *Dunaliela tertiolecta*
Monovinyl chlorophyll *c*3	In acetone: 446,580,624 nm	Minor pigment in haptophytes
Pheophorbide a	In acetone: 410,505,535,559, 608,666 nm	Occurs in senescent algae and in zooplankton fecal pellets and in sediments
Pheophytin a,	In acetone: 410,505,535,560, 610,666 nm	Found in zooplankton fecal pellets, senescent algae, sediments
Pheophytin b,	In acetone: 433,527,597,653 nm	Acid-catalysed demetallation in slightly acidic extracts. Found in Protozoans and plant detritus
Pyropheophorbide a		Occurs in senescent algae and zooplankton fecal pellets
Pyropheophytin a	In acetone: 410,507,536,609, 667 nm	Occurs in senescent algae and zooplankton fecal pellets

Each of the pigments described in Table 1 have a slightly different chemical structures and, in many cases, will also display unique retention times when analyzed by high performance liquid chromatography (HPLC). These different types of chlorophylls can also be diagnostic for specific algal taxonomic groups, e.g., divinyl chlorophyll *a* is unique to numerically dominant prochlorophytes in the open ocean and chlorophyll *c*3 can serve as a tag for haptophytes such as coccolithophorids (Roy et al. 2011).

In addition to chlorophylls, carotenoids are also naturally occurring lipid-soluble pigments found in phytoplankton. These light-harvesting pigments are considered accessory as they help extend the range of wavelengths for photosynthesis and protect phytoplankton from UV radiation and destructive light in the presence of oxygen. Carotenoids provide insight into taxonomic diversity of

phytoplankton communities and serve as diagnostic markers for globally-distributed natural phytoplankton communities.

Measuring Chlorophyll in Water Samples

Although chlorophyll analysis is one of the most commonly applied assays in biological oceanography, there are still confounding factors in current methods that lead to uncertainty in the absolute concentration of chlorophyll *a* in natural planktonic environments. The potential formation of chlorophyllide *a* during extraction is one potential source of error. Chlorophyllide *a* is spectrally identical to chlorophyll *a* on a mole-specific basis, and is therefore not discriminated in the most commonly used bulk fluorescence methods of detection applied to crude extracts of algal pigments (Lorenzen 1967; Welschmeyer 1994). However, HPLC easily discriminates chlorophyllide *a* from chlorophyll *a* on the basis of their widely different polarities. Chlorophyllide *a* is a polar molecule that elutes quickly off a reverse phase HPLC column; under conditions applied in this study it has a shorter elution time of ca. 10-11 min., whereas the non-polar chlorophyll *a* molecule has a longer elution time of ca. 35 min. HPLC's ability to separate other chemical compounds such as carotenoids based on elution times allows us to further examine the chemical makeup of the phytoplankton communities.

Chlorophyllide *a* is often detected in samples, however it is not always clear if the pigment was naturally present in the algal cells prior to extraction or formed as an artifact of the extraction process. Substantial evidence suggests that chlorophyllide *a* is often an artifact of chlorophyll *a* degradation during extraction in organic solvents, especially when the water content of aqueous solvent mixtures is high (Barrett and Jeffrey 1964; Hu et al. 2013, Jeffrey and Hallegraeff 1987). However, it has also been suggested that chlorophyllide *a* may be an indicator pigment attributed to specific physiological stress in marine algae. For instance, some diatoms have been shown to have increased levels of enzymatic chlorophyllase activity resulting in higher cellular concentrations of chlorophyllide *a* (Barrett and Jeffrey 1964). This was also supported by the study of natural spring bloom succession of diatoms off the coast of Sydney, Australia (Hallegraeff 1981).

The detection of chlorophyllide *a* by chromatographic separation can be the result of either 1) extraction artifact, 2) physiological degradation or 3) taxon-specific tendencies to show high chlorophyllide *a* levels (Jeffrey and Hallegraeff 1987). If we are able to eliminate the potential for artifactual production of chlorophyllide *a,* then we can focus our attention on its possible role in the physiology of the cell. An extraction artifact is created in the presence of water, which is a hospitable environment for the chlorophyllase enzyme. If chlorophyllide *a* is a product of physiological stress, it may be inaccurate to include it in productivity metrics such as chlorophyll-specific photosynthetic performance, relative algal biomass levels prediction of photosynthetic rates using chlorophyll-light models (Wernand et al. 2013) and remote satellite sensing of primary production. Specifically, it is presumed that chlorophyllide *a* is not an active participant in organized light absorption energy transfer and therefore should not be included in bulk measures of chlorophyll *a* used in calculating potential photosynthetic rates. While methods such as spectrofluorometry and spectrophotometry can define the dominant pigments in pure form, the discrimination is largely lost in crude extracts. It is essential to determine an optimized method that accurately differentiates pigments and their degradation products and eliminates the artifactual formation of chlorophyllide *a*. In order to accurately measure pigments and degradation products there is a need for a method that physically separates compounds (already available using HPLC) and also minimizes artifactual formation of chlorophyllide *a*.

Metabolically, chlorophyllide *a* is both a degradation product of chlorophyll *a* and is also an intermediate in the biosynthesis of chlorophyll *a*. Chlorophyllide *a* is the last intermediate before chlorophyll *a* in chlorophyll synthesis (Fig. 1a). Chlorophyll-synthase (chl-synthase) catalyses ester bond formation between the precursor chlorophyllide *a* and the phytol chain. As discussed above, chlorophyllide *a* can also be a product of the breakdown of chlorophyll *a* when the enzyme chlorophyllase hydrolyses the ester bond (Hu et al. 2013). Chlorophyllase is found in the inner envelope of the chloroplast (Matile et al. 1996). Chlorophyllase has been extensively studied in higher land plants in relation to ripening or plant senescence

(Daood 2003). There tends to be elevated levels of chlorophyllide *a* and chlorophyllide *b* in the initial growth stages of plants. One of the primary methods for collecting analytical quantities of chlorophyllide *a* is from germination of pumpkin seeds, harvested at the earliest stages of light exposure just before greening of the seedlings (Sundqvist et al. 1979). The chlorophyllase enzyme is also known to be elevated in senescent leaves (Daood 2003). Photosynthetic organisms in the ocean possess similar pigment complexes to higher land plants and there is reason to suspect a similar breakdown pathway of chlorophyll occurs in phytoplankton by the enzyme chlorophyllase (Jeffrey and Hallegraeff 1985).

In addition to its normal occurrence in cells as a precursor or a breakdown product of chlorophyll *a*, chlorophyllide *a* may also form as an artifact during pigment extraction due to the presence of the chlorophyllase enzyme contained within the aqueous organic solvent extracts. Chlorophyllase activity requires water to hydrolyse the ester bond (Fig. 1b). The enzyme chlorophyllase could possibly be activated with cell harvesting techniques with a non-optimal extraction solvent. From previously published work we know that chlorophyllide *a* can be created during extraction with solvents containing high water content such as 50% aqueous acetone (Barrett and Jeffrey 1964; Jeffrey and Hallegraeff, 1987; Hu et al. 2013). In 50% acetone, chlorophyllase is stable, active and creates very high yields of chlorophyllide *a* (Barrett 1964); this characteristic has been exploited as a simple assay for chlorophyllase *a* activity (Jeffrey and Hallegraeff, 1987). Therefore, activity can be increased in aqueous solvents with high water content and can be decreased by extracting pigments in non-aqueous solvents. Barrett and Jeffrey (1964) employed 50% acetone (water:acetone; vol:vol) as a routine extraction process to promote high levels of chlorophyllide *a* in phytoplankton extracts. The common chlorophyll extraction solvent (90% acetone) yields significantly greater total chlorophyll extraction than 50% acetone and is thus the preferred solvent. 100% acetone yields salt precipitation in small volume acetone extracts (<1.5 mL) and is thus problematic in HPLC analysis. Small sample extraction volumes promote increased analytical sensitivity (Welschmeyer, personal communication). Suzuki et al. (2005) applied the protein reductant reagent dithiothreitol (DTT) as an enzyme

inhibitor which, in some cases, yielded lower levels of chlorophyllide *a* formation than in 90% acetone. The effect of changing water content in the extraction solvent mixture on chlorophyllide *a* formation was therefore a carefully monitored parameter in this study.

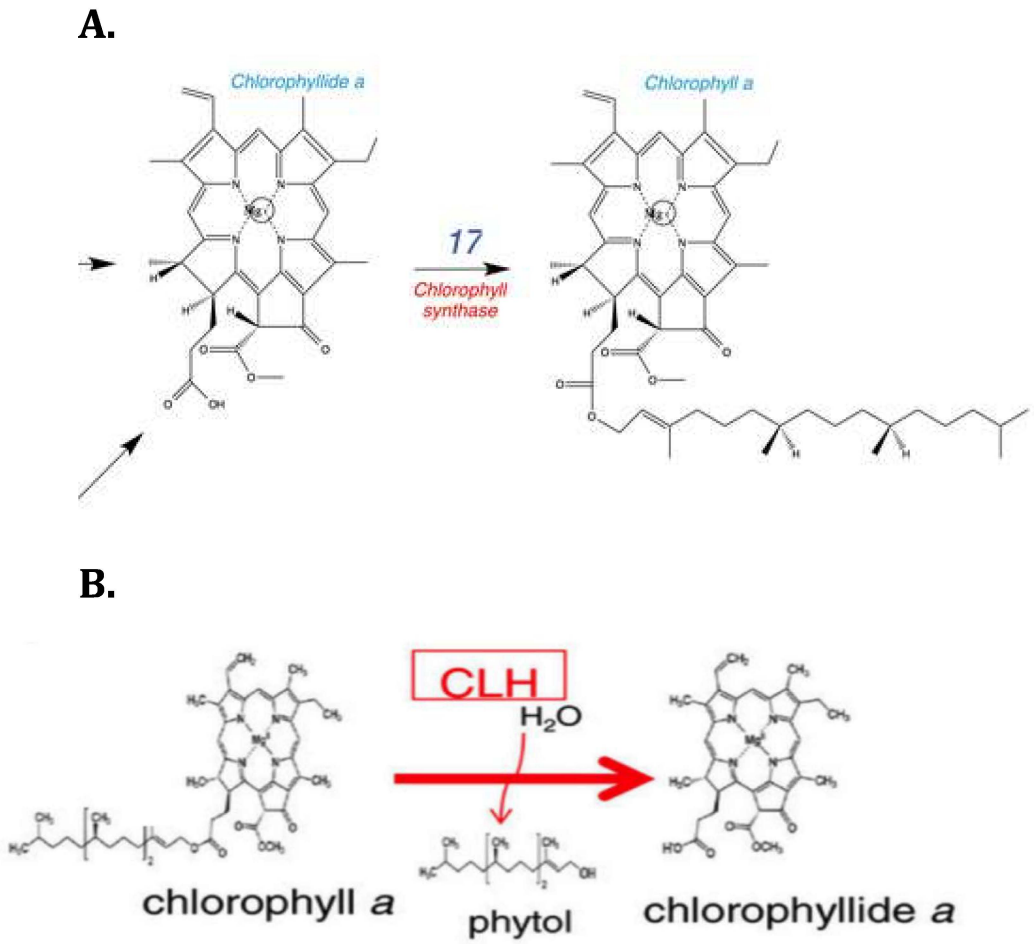


Fig. 1: Chlorophyllide *a* in chlorophyll synthesis and degradation. A) The enzyme chlorophyll synthase transforms the chlorophyllide *a* intermediate into a chlorophyll *a* (adapted from Hu et al. 2013). **B)** The enzyme chlorophyllase (CLH) breaks chlorophyll a into chlorophyllide *a* by hydrolyzing ester bond cleaving off the phytol chain (adapted from Hu et al. 2013).

Microwave-assisted Extraction

An alternative method to denature enzymes is through heating which can be done rapidly through the application of a microwave technique. Chemical assays

now routinely use microwaves as an alternative energy source to enhance reaction rates (Kumari 2017). The primary objective of this study was the optimization of a microwave technique for the purpose of minimizing artifactual chlorophyllide *a* production and maximizing the final yield of pigments (chlorophylls and carotenoids) in a simplified, routine extraction technique (Eskilsson and Björklund 2000).

Microwaving excites the water molecules and this heats the sample, possibly inhibiting chlorophyllase enzyme activity if applied at appropriate levels. In addition, the water quickly turns into a gas phase, resulting in substantial loss of wet weight to the standard GF/F filter harvesting method, thus reducing the water content of the final extract. This can also be helpful in reducing unwanted chlorophyllase activity since Barrett and Jeffrey (1964) showed a clear relation between chlorophyllide *a* levels and water content of various aqueous acetone mixtures. A microwave-based technique thus has the potential to prevent chlorophyllide *a* formation. However, the effect of microwave treatment on pigment integrity is unknown, and this study employed simple control preparations to test whether pigments might be destroyed or, conversely, might be extracted from algal tissue more efficiently. The latter could drive an overall increase in the extraction yield of total chlorophyll (chlorophyll *a* +chlorophyll a'+ chlorophyllide *a*), which implies a potential underestimate of true oceanic chlorophyll *a* concentrations using conventional non-microwave extraction techniques.

The primary objective of this study was the optimization of a microwave technique for the purpose of minimizing artifactual chlorophyllide *a* production and maximizing the final yield of pigments (chlorophylls and carotenoids) in a simplified, routine extraction technique. An ideal goal would be to have a single optimized protocol that would allow for taxonomic insights by maximizing extraction yield for all pigments.

Materials and Methods

Collection of Water Samples

In Fall and Spring 2017 and Fall 2018 water samples were collected in freshwater, brackish and ocean environments in coastal Central California. Water samples were collected in Elkhorn Slough, Moss Landing Harbor, Moss Landing Jetty, and Moss Landing Beach in Moss Landing, CA (Fig. 2). Water samples were also collected in Lake El Estero and Monterey Bay Del Monte Beach in Monterey, CA and Lake Pinto in Watsonville, CA. Open water samples from Monterey Bay were collected on the RV John H. Martin along a routine sampling transect for the Watsonville Water Department.

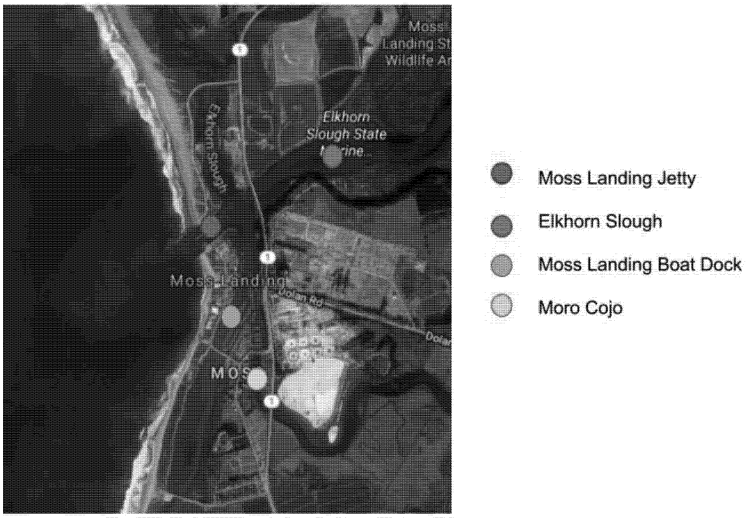


Fig. 2: Water sample locations in Moss Landing, CA.

Extraction of Pigments from Water Samples

Water samples were collected from several marine and freshwater environments and were processed as described below. In order to extract pigments from biological water samples, cells were harvested onto glass fiber filters (Whatman GF/F; nominal 0.7 µm pore size, 25 mm diameter) by vacuum filtration and extracted in 2 mL screw cap microcentrifuge tubes containing 1 – 1.5 mL 90% acetone (pipetted quantitatively). The tubes were then stored in a -20˚C freezer for at least 24 hrs. and then the pigments extracts were prepared for analysis with

HPLC (Zapata et al. 1987). The preparation consisted of vortexing on a 12 position tube carrier (2 min.; highest speed) followed by filter packing with a stainless steel spatula to wring sample filters of pigment extract trapped within the fibers of the filters. The extracts were inverted twice gently to mix the extract volume without dislodging the filter from the bottom of the microcentrifuge tubes. All tubes were then centrifuged at 10,000 rpm (microcentrifuge) to clear suspended filter pulp and desired volumes of each sample were pipetted into clean, screw-cap polypropylene HPLC autosampler vials with air-tight, pierceable septa (Teflon). Solvent soak of filters in 90% acetone is a common pigment extraction method used in biological oceanography. This pigment extraction method is used by long scale oceanographic programs such as JGOFS, HOTS and BATS (Mantoura and Llewellyn 1983, Welschmeyer 1994). For each control filter described above a replicate microwaved filter was placed on an open plate in a conventional Magic Chef microwave oven (1.5KW) and microwaved for 20 seconds at full power immediately after filtration, and then extracted and prepared exactly as above.

Optimizing Microwave Assisted Extraction: Effect of Time and Solvent

Lake El Estero and Moss Landing Harbor water was tested to determine appropriate microwave time. Filters were microwaved for various times in order to determine the optimal conditions for maximizing extraction yield while minimizing additional artifacts from excessive heat. Filters were placed on an open plate in the microwave. Microwave time was evaluated over a range of total microwave exposure times, in increasing 5 second increments. All samples were extracted in 90% acetone, unless otherwise noted. The effect of the microwave was a noticeable expansion of the thickness of each filter as the water evaporated. Gravimetric analysis relative to initial filter dry weight showed that the filters were fully dry after a 20-30 seconds of microwave exposure. A separate experiment compared microwave drying to simple pat-drying with paper towels. Filters were patted dry, with a paper towel and then extracted in 90% acetone as above. Patting the filters dry with a paper towel represents a technique that removes considerable retained

water but would not be expected to denature the chlorophyllase enzyme. The water holding capacity of the GF/F filter is substantial. Gravimetrically, a mean of 0.21 mL water was retained on 6 replicate filters processed with seawater samples under vacuum conditions of 1/3 atm. Thus, the net final acetone concentration of an extract prepared by adding a wetted 25 mm diameter GF/F filter to 1 mL 90% acetone would be approximately 74%. In all cases in this study, the extraction volumes were corrected for the additional water present in the control filters; no correction was needed for the microwaved samples. We performed an acetone dilution experiment as a simple assay for chlorophyllase activity and for determination of the water concentration resulting in maximum chlorophyllide *a* production during extraction. This simple experiment was accomplished by diluting 100% acetone with various pipetted volumes of water, with correction for filter-retained water as appropriate.

HPLC Analysis

Samples were analyzed by HPLC (High Performance Liquid Chromatography, Thermo Fischer Separation Products) using small modifications of the general method by Zapata et al. (2000). A Walters C_8 Symmetry 15 cm column, with 3.5um particles was used as the separation column; the flow rate of the HPLC was 1.0 ml/min. Samples were injected by autosampler maintained at 10°C at all times; the autosampler was programmed to make 1.5x dilutions of extracts with water (1:2, water:extract, vol:vol) just before each injection to prevent band-spreading of the early eluting polar compounds, e.g., chlorophyllide *a*. HPLC sample injections were 200 microliters unless otherwise noted. Chlorophyll *a* and chlorophyllide *a* were quantified by peak area at 665 nm detected on a Thermo UV6000 photodiode array absorbance detector. Chlorophyll *a* had an elution time of ca 35 min; chlorophyllide *a* was found to elute at ca. 10-11 mins. The effect of microwave and non-microwaved (control) treatment filters on chlorophyllide *a* levels and on total chlorophyll was determined by analyzing the peak areas directly with no correction for response factors. Chlorophyllide *a* and chlorophyll *a* standards were quantified assuming equal mole-specific absorptivity and produced response factors (raw area

units/µmole loaded) that were within 10% of each other. All chlorophyllide *a* that was below the detection level of instrument is stated as 0; typical peak areas for detectable peaks ranged from 100,000 to 1,000,000,000 relative area units, undetected peaks were <1,000 area units. Total chlorophyll *a*-like pigments are equal to the sum of chlorophyll *a,* chlorophyll *a*', and chlorophyllide *a*. Final chlorophyllide *a* percent for each sample was calculated relative to the 'total' sum of chlorophyll *a* peak areas, as defined above. Microwaved and non-microwaved (control) samples were normalized for volume changes that resulted from evaporation of water from microwave treated filters (nominally 0.2 mL of water was lost upon microwave treatment; thus, the final extraction volume was 1.5 ml vs. 1.7 ml for microwaved vs. control samples, respectively).

Extraction Temperatures and Liquid Nitrogen Freeze/Thawing Treatments

Liquid nitrogen freeze/thaw procedures represent mechanical disruption methods that have been used to improve extracted pigment yields (Lepesteur et al. 1993) by disruption of cell walls and membranes. Water samples (64 mL) from Lake El Estero were filtered and were processed as described below. Phytoplankton communities from Lake El Estero were chosen for this test based on preliminary results showing higher pigment yields from microwave treatment versus conventional 90% acetone soak in most Lake Estero samples. Harvested cells on 25 mm GF/F filters were analyzed in triplicate using the following procedures: 1) filters were put directly in quantitative volumes of 90% acetone (wet soak) and stored in a -20 degree Celsius freezer for 24 hours prior to HPLC analysis, 2) filters were treated as above but stored in the dark at room temperature (20˚C) for 24 hours prior to HPLC analysis, 3) filters were microwaved for 30 seconds and then extracted in 90% acetone directly and then stored at freezer temperature (-20˚C), 4) filters were microwaved for 30 seconds and then extracted in 90% acetone directly and then stored in the dark at room temperature for 24 hours prior to analysis, 5) filters were put in clean, dry screw-cap microcentrifuge tubes with caps loosely tightened and immersed directly in a Dewar container of liquid nitrogen for 20 seconds, thawed at room temperature for 1 min and extracted in 90% acetone and

stored at freezer temperature -20˚C until analysis , 6) filters were put in clean, dry screw-cap microcentrifuge tubes with caps loosely tightened and immersed directly in a Dewar container of liquid nitrogen for 20 seconds, thawed at room temperature for 1 min and put in 90 % acetone and stored in the dark at room temperature until analysis 7) filters were put in clean, dry screw-cap microcentrifuge tubes with caps loosely tightened and immersed directly in a Dewar container of liquid nitrogen for 20 seconds, thawed at room temperature for 1 min and then microwaved for 30 seconds put in 90 % acetone and stored in the freezer (-20˚C) until analysis 8) filters were put in clean, dry screw-cap microcentrifuge tubes with caps loosely tightened and immersed directly in a Dewar container of liquid nitrogen for 20 seconds, thawed at room temperature for 1 min and then microwaved for 30 seconds put in 90 % acetone and stored in the dark in room temperature(20˚C) until HPLC analysis.

Cultured Phytoplankton

A time series growth experiment was performed to determine if chlorophyllide *a* was produced naturally at all times in phytoplankton or specifically as a result of senescence in late phase culture growth. The phytoplankter *Phaeodactylum tricornutum* (obtained from CCMP Bigelow Laboratories, Bar Harbor, Maine) was cultured for the purpose of this study. Sterile nutrient-enriched seawater growth media was prepared in order to support the culture. Seawater from Monterey Bay from the running seawater system of Moss Landing Marine Labs was filtered through 0.2 µm filters, autoclaved and augmented with nutrients as per Guillard's F/2 formulation (882 µM nitrate, 106 µM silicic acid, 36 µM phosphate, vitamins, trace metal). Five ml of F/2 media was inoculated with *Phaeodactylum tricornutum* culture in glass 5 mL sterile test tubes. After inoculation, cultures were grown in a 24 hour continuous-light 15˚C incubator. Growth was monitored as relative chlorophyll fluorescence units (RFU) using the culture tubes as cuvettes in a 10 AU fluorometer (Turner Services) outfitted with optical filters and excitation lamp described by Lorenzen (1967). Exponential growth phase was identified from linear increases in the log of chlorophyll fluorescence. Growth rates (µ) were

calculated from the exponential growth model, $Nt = N_0 e^{\mu t}$. All the cultures were monitored over time to identify mid-exponential phase and then sub-cultured by inoculation into new tubes of fresh media. By repeated sub-cultures in mid-exponential phase we were able to obtain a consistent growth rate ensuring full acclimation to nutrient saturated conditions. Once acclimated growth was established, *Phaeodactylum* was transferred into 12 tubes of fresh media for a final time to follow pigmentation changes over time as the cultures entered the stationary phase of batch culture growth. All tubes were grown under identical conditions. Six of the tubes were harvested at mid-exponential phase (day 4) to represent nutrient saturated growth and 6 were harvested at stationary phase (day 11) to represent stressed conditions. For each set of six tubes (5 mL culture volume in each) the cultures were volumetrically filtered onto 25 mm diameter GF/F filters. Three filters were controls (non-microwaved) and 3 were treated (microwaved for 20 seconds) prior to extraction with 1.5 ml of 90% acetone. These extracts were stored in a freezer for one week followed by High Performance Liquid Chromatography (HPLC) analysis. Sample volumes of 200 µL were injected for all phases of the culture.

Time Series Analysis of Algal Cultures

Four species of phytoplankton were cultured over time with natural sunlight at room temperature on a north-facing laboratory window sill at Moss Landing Marine Laboratories (MLML). The genera included *Thalassiosira Weissflogii* (a diatom), *Tetraselmis* (a green alga), Unknown Diatom, and *Scenedesmus* (a green alga). *Thalassiosira weissflogii* was obtained from the Kudela Lab at University of California Santa Cruz (UCSC). *Scenedesmus* and *Tetraselmis* was obtained from Carolina Biological Supply. The unknown diatom isolate was isolated from Monterey Bay water at Moss Landing Marine Labs. Cultures were 9, 22, 34, 51, 64 and 79 days old at the time of harvest. Cultures (5 mL) were all filtered onto glass fiber filters (Whatman GF/F; nominal 0.7 µm pore size). For each time series sample, filters were placed on an open plate in the microwave for 20 seconds and corresponding

replicates were not microwaved to serve as controls. Samples were analyzed using HPLC as described above.

Results

Reported below are studies to optimize the microwave-assisted extraction technique. The parameters investigated included 1) total chlorophyll extracted (extraction yield) and 2) proportions of chlorophyllide *a* relative to all chlorophyll *a*-like pigments (total chlorophyll *a;* defined as chlorophyllide *a* + chlorophyll *a* + chlorophyll *a*'). The optimization experiments included samples from (a) various marine and lake environments, and (b) single species grown in pure culture. Finally, the optimized extraction method was applied to a survey of samples, both natural and cultured, to compare standard to microwave-assisted chlorophyll extractions.

(1) Optimization of Microwave-Assisted Extraction: Effect of Microwave Time

Figure 3 shows the reduction in chlorophyllide *a* content (relative to total chlorophyll *a*) as a function of microwave time in samples from four locations in the coastal Monterey area. We found that 20-30 seconds was an optimal time of microwaving for reduction of chlorophyllide *a* (Fig. 3). In the Lake El Estero samples, 72% of the total chlorophyll was detected as chlorophyllide *a* in the untreated control, and that was reduced to <0.01% after microwaving for 20 seconds (Fig. 3). Similar results (<0.01% chlorophyllide *a*) were seen in samples from other locations including Moss Landing harbor at high tide and low tide and Moro Cojo slough.

Total chlorophyll *a*-like pigments increased from 2.3-4.4x in the Lake El Estero samples when microwaved for 20 seconds (e.g. Fig. 4, green). A more modest increase in total chlorophyll yield of about 1.86x was found in Moss Landing Harbor (shown in blue; Fig. 4).

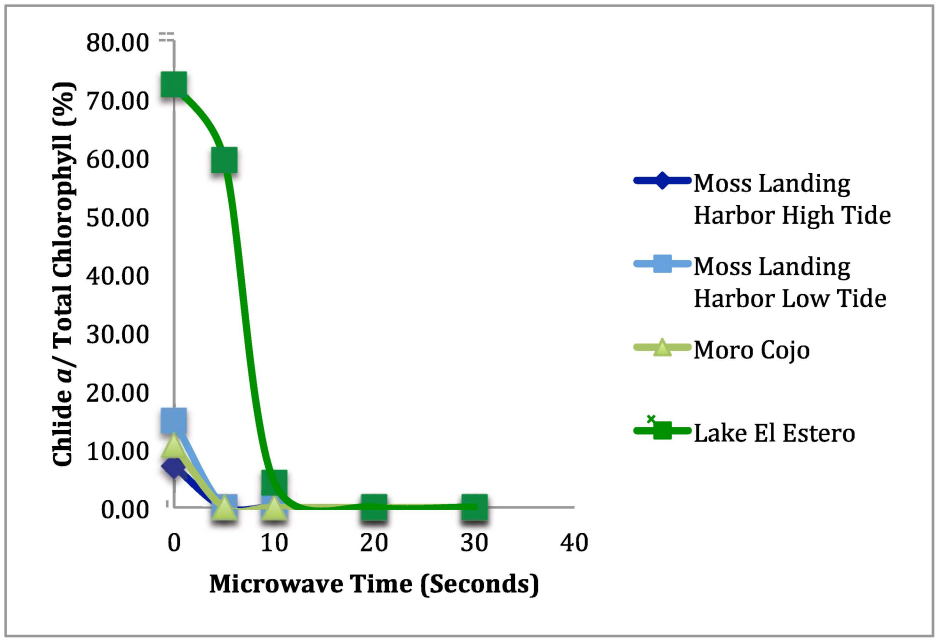


Fig. 3: Chlorophyllide *a* % in response to different microwave times in samples from various locations. Chlorophyllide *a* percentage (chlorophyllide *a*)/ total chlorophyll). Total chlorophyll =Chlorophyll *a* + Chlorophyll *a*'+ Chlorophyllide *a*. All locations are located in area of Monterey/Moss Landing, CA.

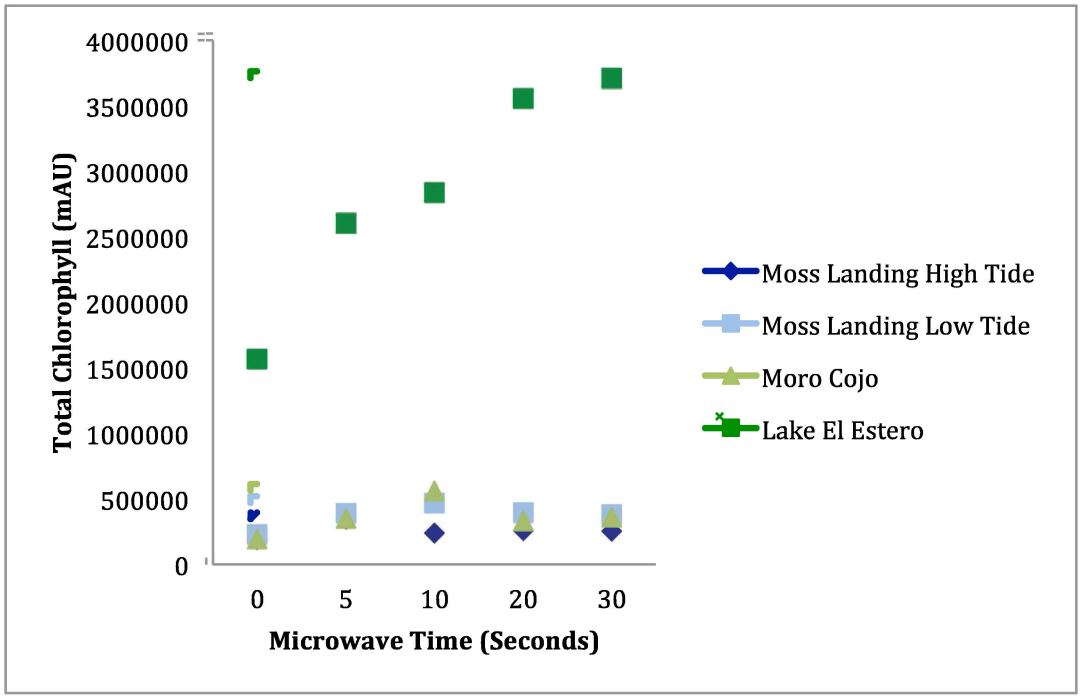


Fig. 4: Total chlorophyll (in mAU) after different microwave times. All samples were extracted in 90% acetone. Note that for the chlorophyll rich samples (Lake El Estero) the HPLC injection volume was reduced from 200 to 100 microliters.

(2) Optimization of Microwave-Assisted Extraction: Effect of Extraction Solvent

Here we confirm the undesirable effect of high water content in aqueous acetone mixtures with respect to artifactual formation of chlorophyllide *a*, as shown previously by Barrett and Jeffrey (1964), Jeffrey and Hallegraeff (1987) and Hu et al. (2013). Standard acetone soak extraction showed marked reductions in chlorophyllide *a* (as % of total chlorophyll *a*) at approximately 85% acetone; at 70% acetone almost 90% of the total chlorophyll *a*-like pigments were present as something other than chlorophyll *a*, e.g. chlorophyllide *a* (Fig. 5a, blue bars). In contrast, note in Fig. 5a that under microwave-assisted extraction the % chlorophyll *a* remains above 80% even in 65% acetone; microwave-assisted extraction yielded <5% chlorophyllide *a* in all acetone water mixtures in the range 75-100%. Our experiments confirm that extractants with high water content (i.e. 50:50 acetone:water) had increased artifactual formation of chlorophyllide *a* (Fig. 5b, blue bars); at that level, microwave-assisted extraction was unable to inhibit chlorophyllide *a* production (Fig. 5b). We conclude that there is still chlorophyllase activity even in 90% acetone. Jeffrey and Hallegraeff (1987) suggested that this problem could be solved by employing a chlorophyllase inhibitor. In this study we have inhibited the chlorophyllase enzyme through the application of heat using a conventional microwave oven.

In Fig. 6 the results for the same experiments described above are plotted to show the pigment extraction yield as 'total chlorophyll *a*-like pigments'. In all cases there was improved extraction yield for all chlorophyll *a*-like pigments when using microwave-assisted extraction (Fig. 6). Total chlorophyll extraction yield was increased, relative to control soak extraction by at least 2x in all of the microwaved samples using >75% acetone (Fig. 6).

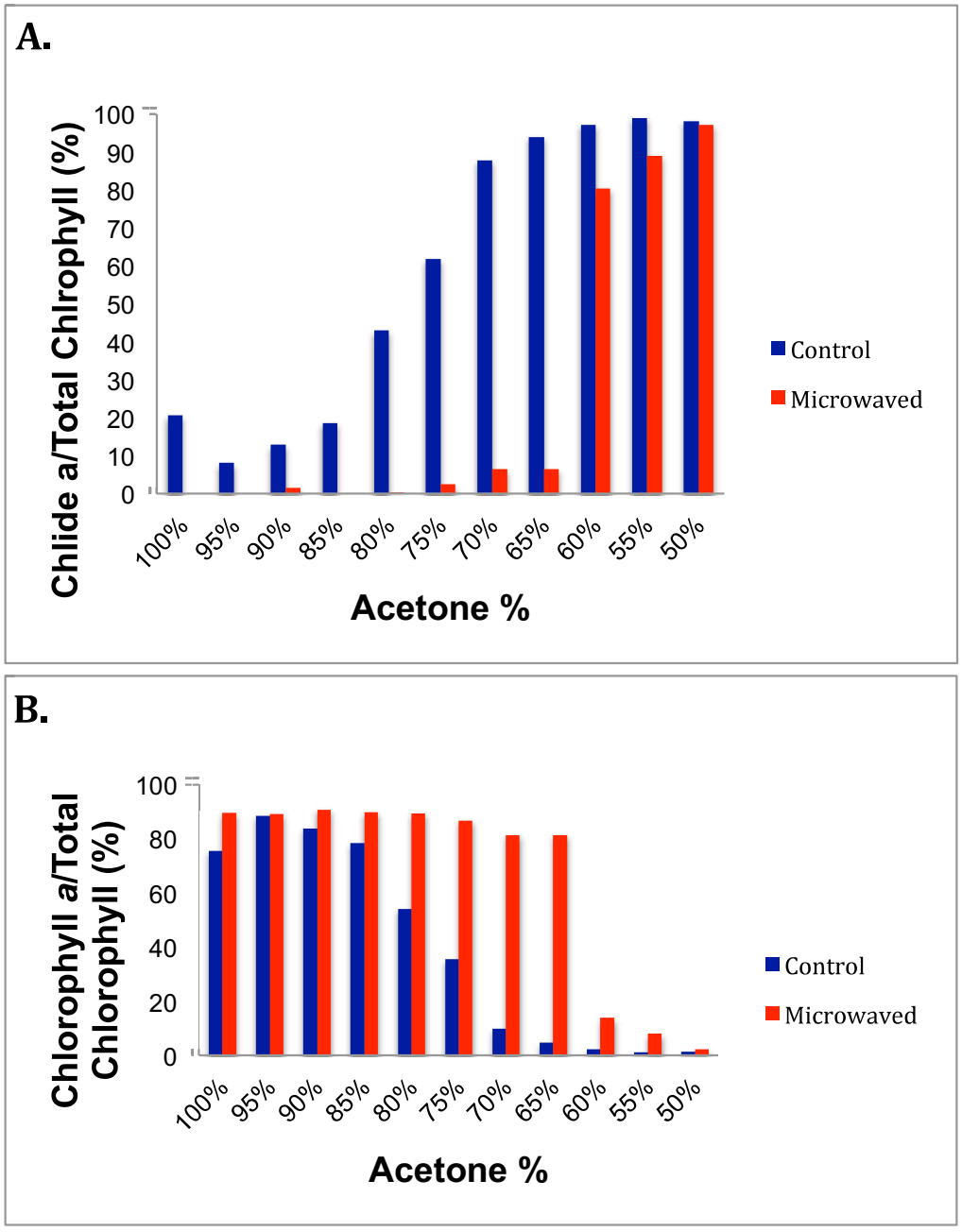


Fig. 5: Chlorophyllide *a* and chlorophyll *a* (%) with different acetone concentrations. A) Chlorophyllide *a* % of total chlorophyll *a*-like pigments in a range of acetone %. **B)** Chlorophyll *a* % of total chlorophyll in a range of acetone %.

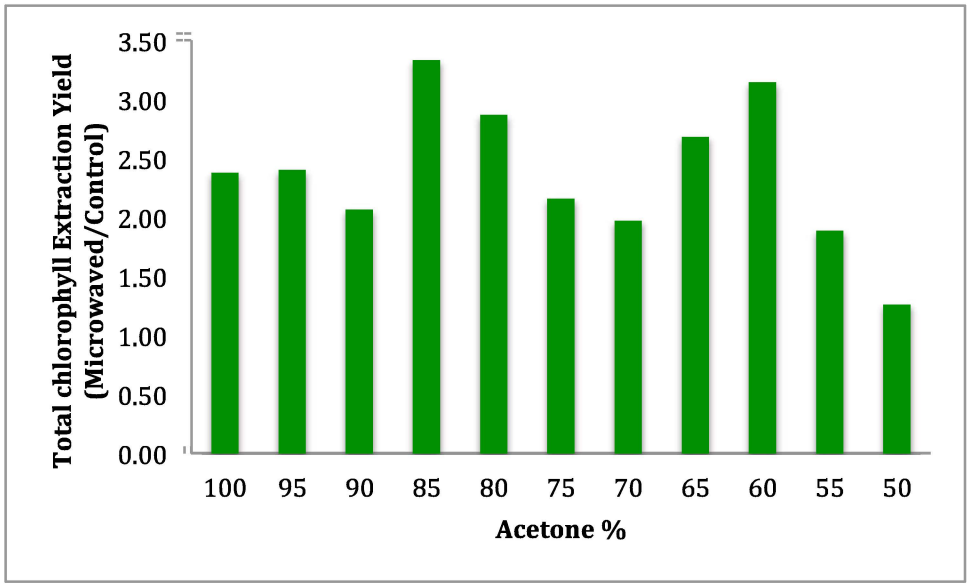


Fig. 6: Total Chlorophyll Extraction Yield with Microwaving. Total chlorophyll *a*-like pigment extraction yield for microwaved samples relative to controls (Microwaved/Control) as a function of various aqueous acetone mixtures.

(3) Optimization of Microwave-Assisted Extraction: Effect of Filter Extraction Temperature

Another aim of this study was to determine the optimal extraction temperature for filters in 90% acetone. The common practice is to store extracts in the dark at the coldest temperatures available to avoid pigment degradation. An experiment was done in order to determine if the microwave technique reduced the degradation of chlorophyll *a* into chlorophyllide *a* at different extraction temperatures. The results (Table 2) revealed that the chlorophyllase enzyme still breaks down chlorophyll, even when filters were extracted in a freezer using control soaking technique. When the microwave technique is applied, chlorophyllide *a* is reduced from 27% to 0.4% of total chlorophyll *a*-like pigments (Table 2). The experiment also revealed that the microwave technique led to a 2.5x increase in extraction yield under freezer conditions (Table 2).

Table 2: Effect of Extracting filters with 90% Acetone at Different Temperatures (measured after 4 days of extraction).

	Treatment	**Total Chl (chl *a* + chlide *a* + chl a')**	**Extraction Yield (Microwave/ Control)**	**Chlorophyllide *a* %**
Freezer	Control	970,022	2.5	26.98
	Microwaved	2,467,009		**0.433**
Fridge	Control	1,290,682	1.6	21.12
	Microwaved	2,129,498		**1.447**
Room Temperature	Control	1,747,132	1.4	26.69
	Microwaved	2,533,288		**1.23**

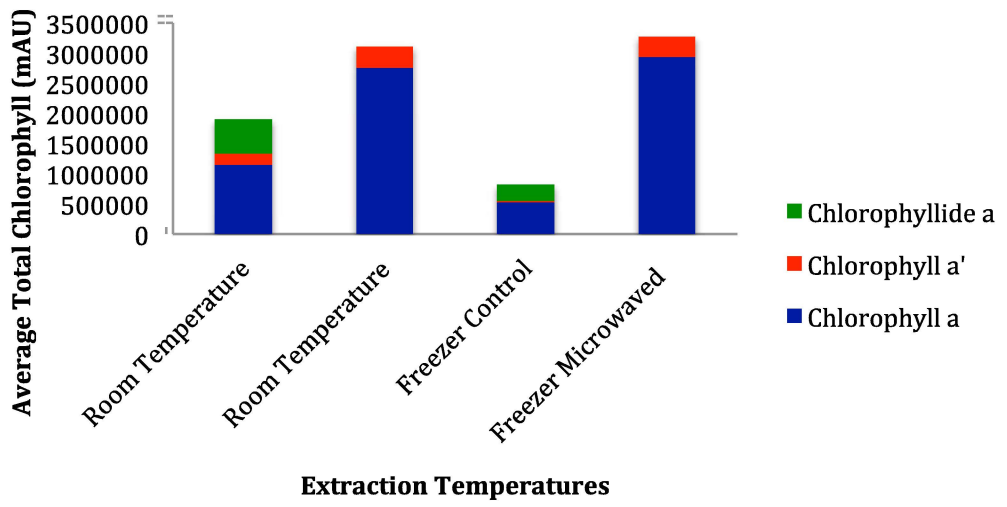
Initial sums of all chlorophyll *a*-like pigment peak areas (in milli-absorbance area units) in microwaved and control samples are shown in Table 2. Microwaving the filters improved extraction of chlorophyll *a* (Fig. 7a, blue) and chlorophyll *a*' peak areas (Fig. 7a, red) in both the freezer and the room temperature extractions; chlorophyllide *a* was no longer detectable in microwaved samples (Fig. 7a, green).

The total chlorophyll *a*-like pigment yield in the control "soak" samples were highly variable depending on the extraction temperature. An ANOVA test indicated that there was a significant difference between extraction temperatures. Since the ANOVA test indicated there was a significant difference, a Tukey-Kramer test of significance was then done, which confirmed that the total chlorophyll *a*-like pigments were significantly different ($p<0.05$) between extraction temperatures in the control samples (Fig. 7b blue). When the microwave technique was applied, it improved extraction of total chlorophyll *a*-like pigments independent of where the samples were stored (in all extraction temperatures) (Fig. 7b). An ANOVA test revealed that there was not a significant difference ($p>0.05$) in the total chlorophyll extracted from microwaved filters in different extraction temperatures. When the microwave samples were compared with the control samples there was a significant difference ($p<0.05$) in all extraction temperatures. Samples that were stored in the

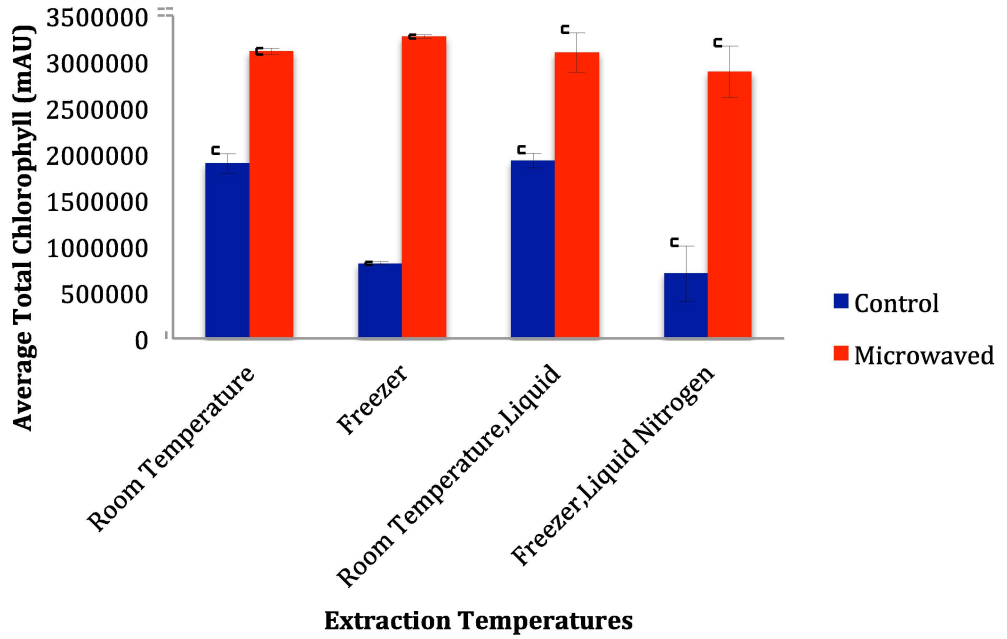
freezer (-20˚C) appear to have the most improved extraction yield. When the microwave technique was applied, it improved extraction yield up to 4.03x compared to the control (Fig. 7c). At room temperature, extraction yield improved by almost 2x with the microwave technique. The microwave technique greatly reduced the degradation of the chlorophyll *a* into chlorophyllide *a*. Chlorophyllide *a* was approximately 30-40% of total chlorophyll in all control samples in the different extraction temperatures. After the application of the microwave technique, chlorophyllide *a* was undetectable (Fig. 8).

Liquid nitrogen freeze-thaw technique has been proposed as an improved extraction methodology due to its simplicity relative to mechanical extraction techniques (e.g., tissue grinding, sonication). Liquid nitrogen has been used to stop enzyme activity with extremely cold temperatures and to break open cells in order to better extract pigments. Our results indicate that the liquid nitrogen did not significantly ($p>.05$) increase the extraction yield of total chlorophyll *a*-like pigments. There was not a significant difference ($p>0.05$) between the freezer temperature soaking extraction and freezer extraction preceded by liquid nitrogen freeze/thaw treatment for 20 seconds. There was also not a significant difference between the room temperature extraction and room temperature with a liquid nitrogen treatment for 20 seconds. The microwave treatment increased the extraction yield of total chlorophyll for samples stored in the freezer by 4.03x. The microwave treatment increased the extraction yield for liquid nitrogen by 4.11x (Fig. 7c). We conclude that the liquid nitrogen appeared to have little effect on improving the extraction yield of total chlorophyll *a* like pigments. An interesting outcome of the test scenario above was that room temperature soaking extraction (without microwave treatment) yielded substantially more total chlorophyll than freezer soaking, the commonly recommended protocol in the JGOFS standard operating procedures (Knap et al. 1996).

A.


Average Total Chlorophyll (mAU)
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Room Temperature
Room Temperature
Freezer Control
Freezer Microwaved
Chlorophyllide a
Chlorophyll a'
Chlorophyll a
Extraction Temperatures

B.


Average Total Chlorophyll (mAU)
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Room Temperature
Freezer
Room Temperature,Liquid
Freezer,Liquid Nitrogen
Control
Microwaved
Extraction Temperatures

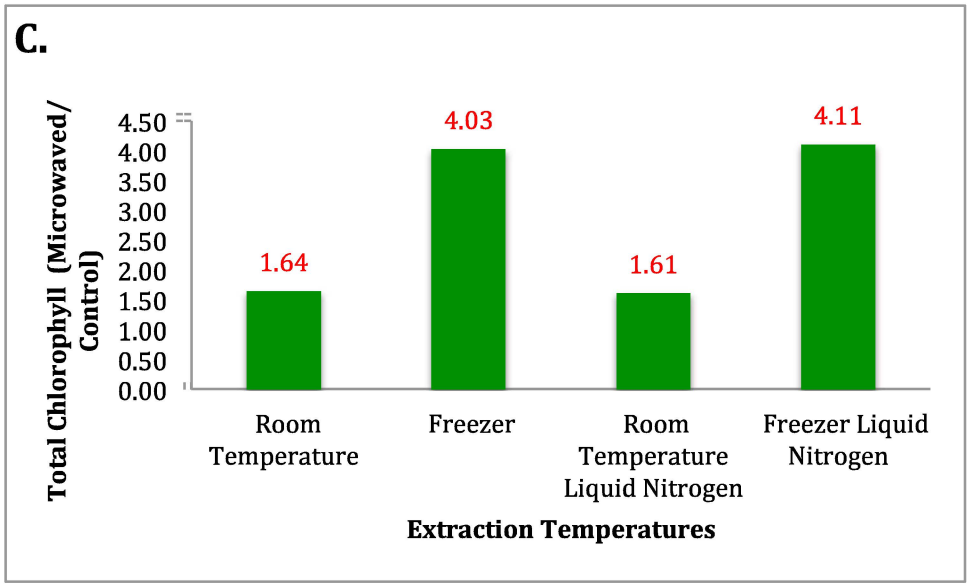


Fig. 7: Effect of extraction temperature on chlorophyll measurements using the microwave technique. A) Composition of total chlorophyll (chlorophyll *a* + chlorophyll *a*'+ chlorophyllide *a*) in different extraction temperatures. Means of each variant of chlorophyll a were calculated from environmental triplicates for each treatment. The injection volume was 150 microliters for all samples. **B)** Average total chlorophyll *a*- like pigments in different temperature conditions. Peak areas (mean ±SD) were calculated by taking the average of total chlorophyll in triplicate environmental samples. The injection volume was 150 microliters for all samples. **C)** Ratio of total chlorophyll *a*-like pigments extraction yields microwave/control with different temperature treatments. Total chlorophyll was calculated for triplicates for each temperature condition. An average was calculated for each of the temperature condition and then the microwaved total was divided by the control to determine how much the microwaving improved extraction yield.

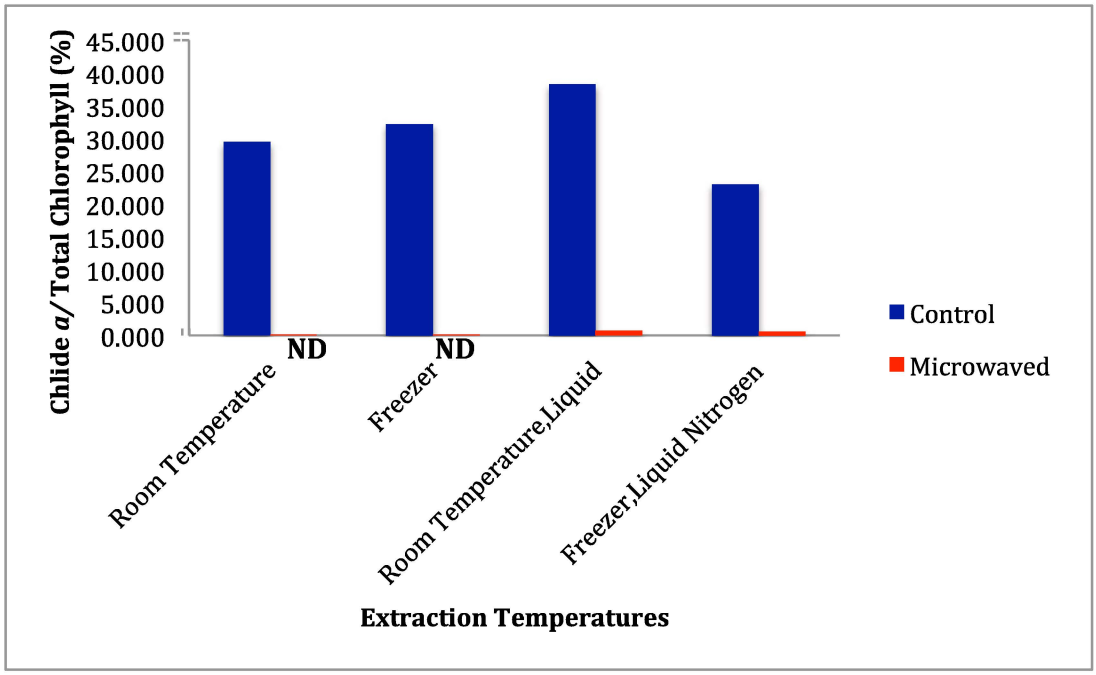


Fig. 8: Average chlorophyllide *a* % in control compared to microwaved samples in different temperature treatments. Chlorophyllide *a* % was calculated relative to total chlorophyll *a*- like pigments. ND= Chlorophyllide *a* % was below detection level.

(4) Application of Microwave- assisted extraction to samples from marine and freshwater environments

To determine the effect of microwaving on pigment yields, the sum of the peak areas of the total chlorophyll in each microwaved samples was divided by the corresponding sum of peak areas in control samples from each of the two different sample locations, freshwater and marine. The extraction yield for total chlorophyll was 2.30x better with the microwave technique in Lake El Estero and 1.34x better with the microwave technique in Moss Landing Harbor water (Table 3). The microwave technique yields the most total chlorophyll (here defined as chlorophyll *a* + chlorophyll *a*'+ chlorophyllide *a*) for both sampling sites (Table 3). Filters treated by microwaving yielded more total chlorophyll in both locations even when compared to the patted dry technique (Table 3).

Table 3: Total chlorophyll extraction yield in control soak samples vs. paper towel patted dry filters. The increase in extraction yield is expressed as the ratio of microwave treated samples to control soak samples.

Location	Microwave/Control	Patted Dry/Control
Lake El Estero	2.30	1.52
Moss Landing Harbor	1.34	1.05

Previous literature had concluded that some algal taxa were characterized by inherently high intracellular chlorophyllide *a* content, particularly in some diatom species (Jeffrey 1974). To determine the differences in chlorophyllide *a* content between marine and lake phytoplankton we applied our optimized microwave technique to a survey of environmental samples (freshwater and saltwater) and cultured phytoplankton as well. The microwave technique improved total chlorophyll extraction yield in the mostly freshwater environment of Lake El Estero by 3.2x. (Fig. 9a). Extraction yield was also improved in Monterey Bay and Moro Cojo. When comparing marine environmental samples to lake and estuary environmental samples the increase in total chlorophyll is more apparent in freshwater or brackish environments (Fig. 9a). As an example, on February 27, 2018 Lake El Estero was 0.76 ppt (mostly freshwater) and Moss Landing Harbor was 30.76 ppt (mostly saltwater). Overall, the microwave technique improved or was equal to the extraction yield of total chlorophyll *a* like pigments in all environments (Fig. 9a).

The freshwater phytoplankton community of Lake El Estero showed particularly high chlorophyllide *a* % using conventional solvent soak technique, with up to 89% of the total chlorophyll *a*-like pigments detected as chlorophyllide *a* (Fig. 9b, blue bar). When the microwave technique was applied chlorophyllide *a* was reduced to undetectable levels (Fig. 9b, red bar). Pinto Lake, a small freshwater body in the city of Watsonville provides an additional example of the ability of the microwave treatment to reduce chlorophyllide *a* formation. In Pinto Lake, 74% of total chlorophyll was detected as chlorophyllide *a,* and when microwaved this was reduced to 1.63%. Since the microwave technique can completely eliminate

chlorophyllide *a* in many samples we hypothesize that the 1.63% chlorophyllide *a* seen here was present in these cells prior to extraction.

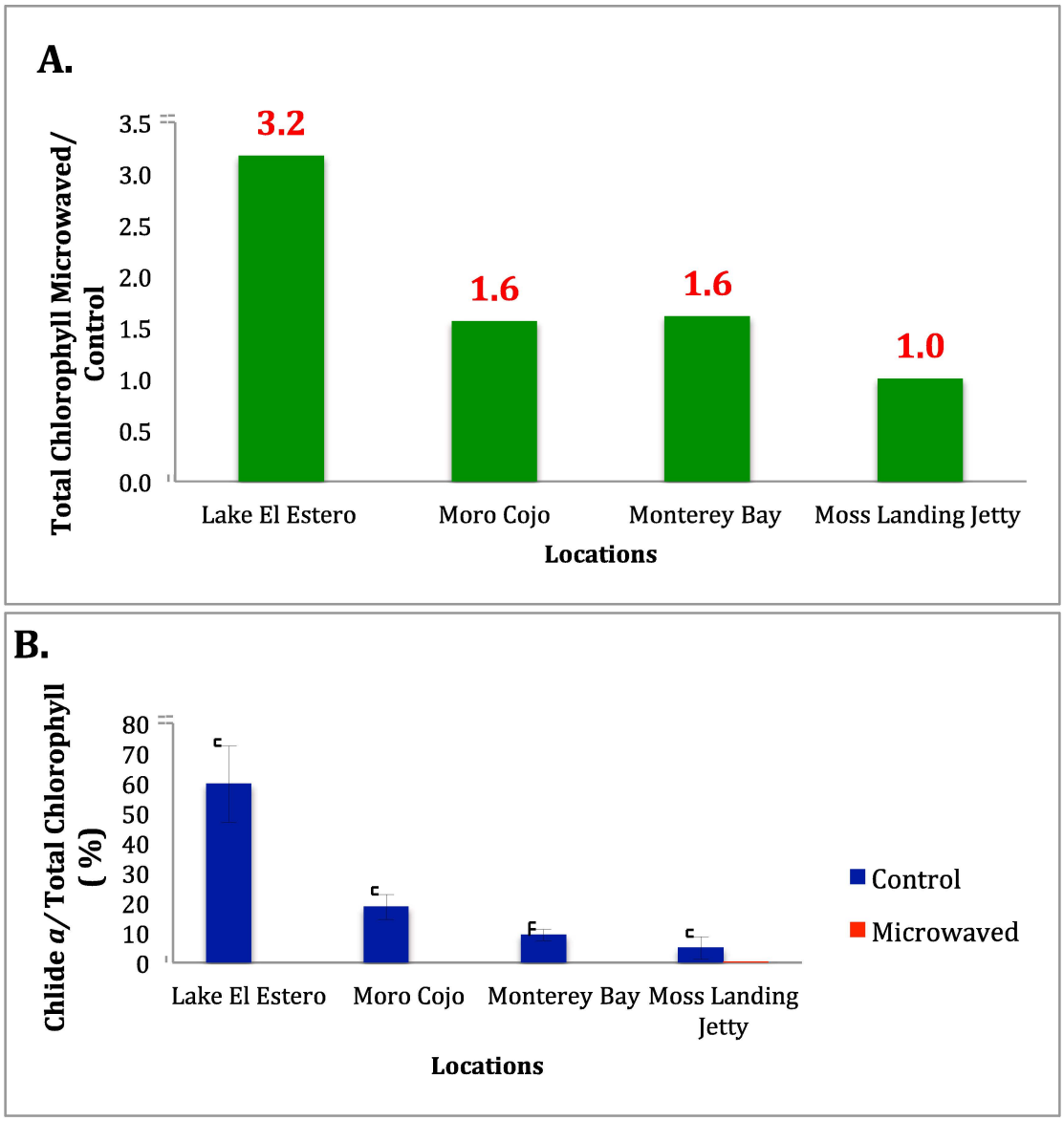


Fig. 9: Chlorophyll extraction yield and Chlorophyllide a % in samples from 4 local environments. A) Chlorophyll extraction yield expressed as the ratio of microwave treated samples to control soak samples from 4 local environments. Total chlorophyll was expressed as the sum of chlorophyll *a* + chlorophyll *a*'+ chlorophyllide *a*. Totals were calculated as an average of samples measured at each site. **B)**Average Chlorophyllide *a* % in control and microwaved samples in different environments. Chlorophyllide *a* % (Chlorophyllide *a*)/ (Chlorophyll *a* +Chlorophyll *a*'+Chlorophyllide *a*). Chlorophyllide *a* % (mean±SD) determined from triplicate assays. Note that chlorophyllide *a* was detectable in microwaved samples only in Moss Landing Jetty.

The mean chlorophyllide *a* percentage out of total chlorophyll in surface samples along a 12 mile transect in Monterey Bay between Watsonville (the mouth of Pajaro River) and the City of Marina is plotted in Fig. 10. When the microwave technique was applied to Monterey Bay samples, the chlorophyllide *a* content in the microwaved samples (1-2%) was greatly reduced relative to control samples (35-75%), but still detectable (Fig. 10). We assume that small levels of chlorophyllide *a* detection after microwave treatment in Monterey Bay may represent extant, in situ concentrations of cellular chlorophyllide *a* (Fig. 10). In other cases, however, chlorophyllide *a* was reduced to undetectable levels after microwave treatment (Fig. 9b). The microwave technique, by eliminating most of the artifactual chlorophyllide *a*, represents a more accurate measurements of cellular chlorophyllide a in the sample. Total chlorophyll extraction yield increased in all environmental samples tested.

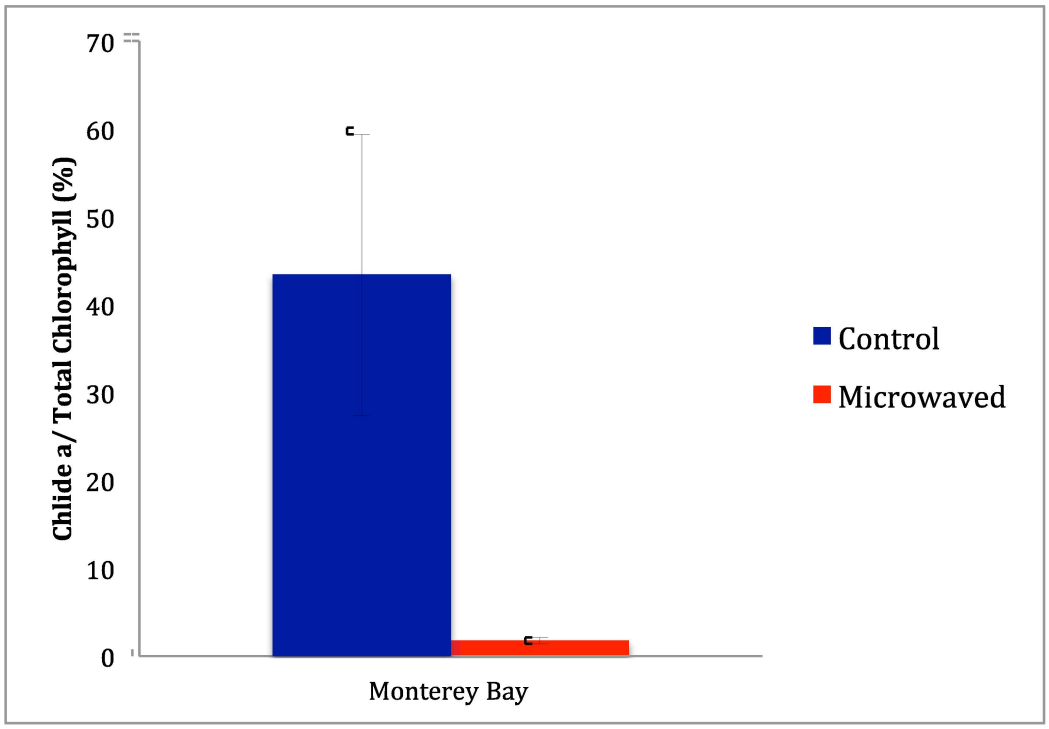


Fig. 10: Microwave effect on Chlorophyllide *a* % in Monterey Bay.
Chlorophyllide *a* % (Chlorophyllide *a*/ Chlorophyll *a*+Chlorophyll *a*'+ Chlorophyllide *a*) expressed as mean ±SD (N = 12)Data from a 12 mile shoreline transect cruise from Watsonville (Pajaro River) to the city of Marina, CA collected on the RV John H. Martin, Moss Landing Marine Laboratories research vessel.

Chlorophyllide *a* in Cultured Phytoplankton Samples

Experimental efforts, using controlled growth of cultured phytoplankton, were made to test the hypothesis that detectable levels of chlorophyllide *a* (after microwave treatment) might be associated with the physiological status of nutrient depleted culture conditions. Previous literature suggested that higher chlorophyllide *a* content was found in senescent (stationary phase) diatoms (Hallegraeff 1980). Experiments utilizing the transition of algal cultures from exponential to stationary growth phases are presented below.

In exponential phase, *Phaeodactylum* growth rates were very consistent among all 12 tubes (Fig. 11). An ANOVA test indicated that there was not a significant difference ($p>.05$) between the growth rates of the 12 tubes in exponential phase. All the samples (1-12) grew equally well in exponential phase with rates of: tubes 1-3 (Growth rate=1.12 ± 0.02), 4-6 (Growth rate=1.15 ± 0.05), 7-9 (Growth rate=1.19 ± 0.03) and 10-12 (Growth rate=1.12 ± 0.04). Tubes 1-6 were harvested in exponential phase (Fig. 11a) and tubes 7-12 were harvested in post-exponential phase after growth had slowed (Fig. 11b). These near stationary phase cultures had almost identical cell density. The average rates during post-exponential phase were: 7-9 (Growth rate=0.19) and 10-12 (Growth rate=0.21). Our microwave technique also increased total chlorophyll *a* extraction yield in both the mid-exponential (1.3x) and stationary (1.5x) phase of the culture (Fig. 12). Microwaving filters from either exponential or stationary cultures resulted in a statistically significant ($p<< 0.05$) decrease in chlorophyllide *a* percentage. However, there was not a statistically significant difference between the same treatments, but different phases (exponential and stationary)($p=0.1$) (Table 4). Both exponential and stationary cultures of phytoplankton contained about the same percentage of chlorophyllide *a* in control (non-microwaved) samples (Fig.13). With the microwave assisted extraction technique low levels of chlorophyllide *a* were seen in cells in both growth phases. The observation that there was very low chlorophyllide *a* levels in stationary phase argues against the suggestion that chlorophyllide *a* formation occurs more in senescent cultures (Hallegraeff 1981).

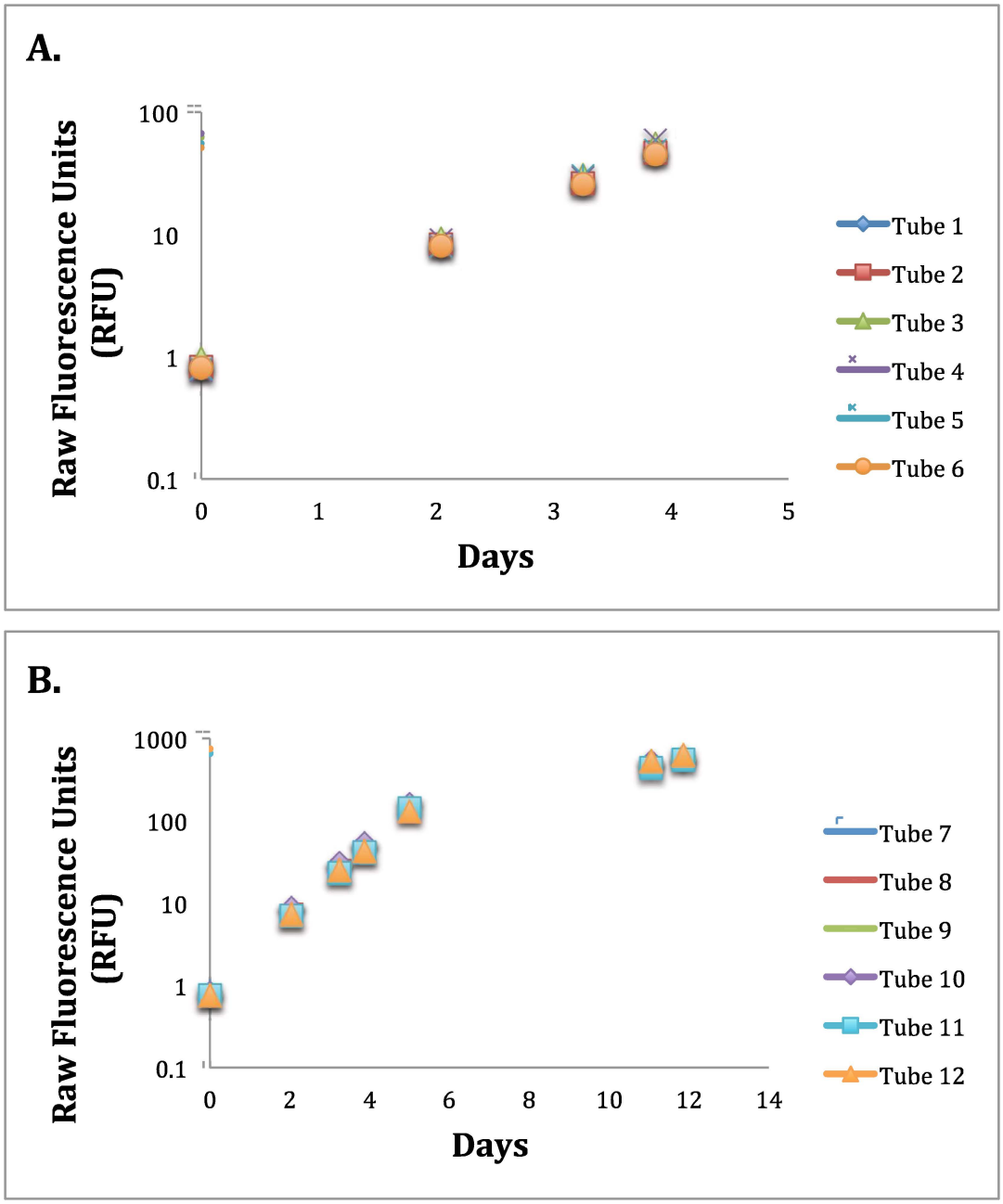


Fig. 11: *Phaeodactylum* culture growth curves. The growth rates for the acclimated cultures were: **A)** Six tubes were harvested in exponential growth at 3.86 days. **B)** Six tubes were harvested in the post-exponential phase at 11.86 days.

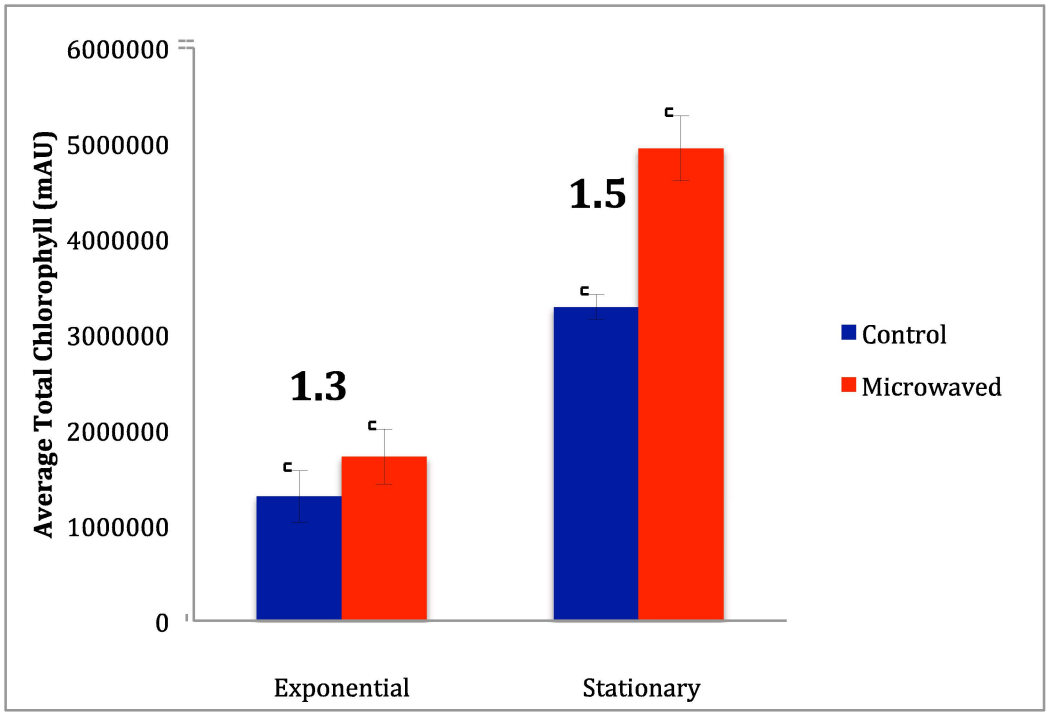


Fig.12: Total Chlorophyll *a*- like pigments in *Phaeodactylum tricornutum* in mid-exponential and stationary phase. Amount of total chlorophyll (peak areas in mAU) that is obtained from the control vs. microwaved filters for the 12 culture tubes (6 were harvested in mid exponential and 6 were harvested in stationary phase). Ratio of average total chlorophyll *a* microwaved/control indicated above bars.

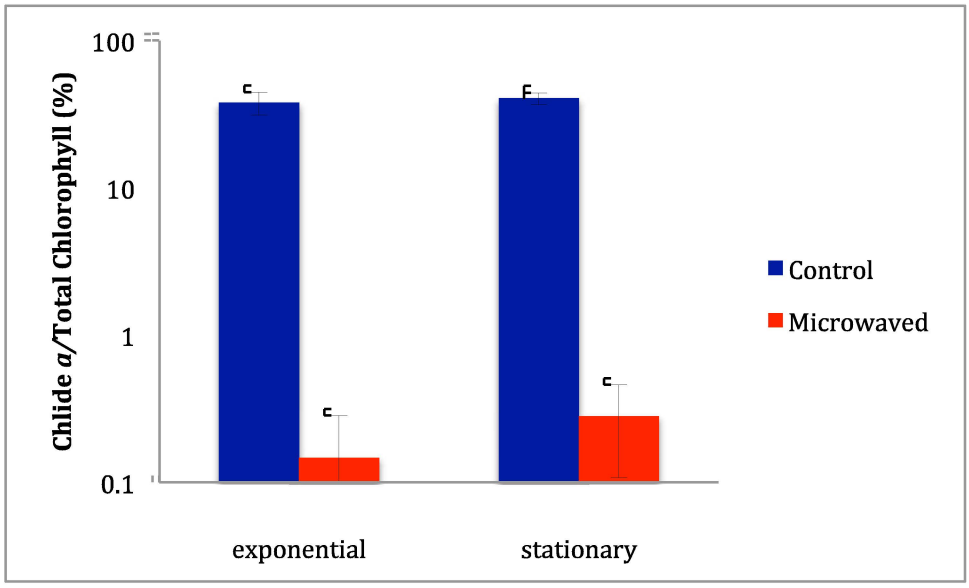


Fig. 13: Chlorophyllide *a* % in the exponential and stationary phase of the *Phaeodactylum* culture as measured in control vs. microwaved samples. The microwaved technique greatly reduced chlorophyllide *a* in the mid exponential and stationary phases of the culture growth.

Table 4: Statistical summary of Tukey Test: The effect of microwave on chlorophyllide a% in stationary and mid-exponential phase of Phaeodactylum culture.* indicates significance $p<0.05$.

Phases	P value
Mid-Exponential-NM -Mid-Exponential-M	0.0000063*
Stationary-M-Mid-Exponential-M	0.9999928
Stationary-NM-Mid-Exponential-M	0.0000007*
Mid-Exponential-NM -Mid-Exponential-NM	0.0428644
Stationary-M-Mid-Exponential-NM	0.0000049*
Stationary-NM-Mid-Exponential-NM	0.5273913
Stationary-M-Mid-Exponential-NM	0.0000065*
Stationary-NM-Mid-Exponential-NM	0.1155381
Stationary-NM-Stationary-M	0.0000007*

Time Series Analysis of Algal Cultures

A survey of 4 algal cultures, held in growth tubes up to 80 days, was made to check for potential taxon-specific traits in % chlorophyllide *a* over prolonged periods of batch culture growth (e.g. , extreme nutrient stress) (Table 5). Chlorophyllide *a* % (chlorophyllide *a*/ total chlorophyll) was examined in four different types of phytoplankton in culture over a 2 month period. *Tetraselmis* (a green alga) had very little chlorophyllide *a* in the control but the microwave technique still worked to reduce the chlorophyllide *a* percentage (Table 5). In comparison there was a high percentage of total chlorophyll detected as chlorophyllide *a* when the samples were not microwaved in the other species tested (Table 5). In the control the older cultures had more chlorophyllide *a* (Table 5). However, when the microwave technique was applied the chlorophyllide *a* decreased so that it was detected at low amounts in all genera. This indicates that the high amount of chlorophyllide *a* seen in those genera might have been an artifact of the extraction (Table 5). The chlorophyllide *a* percentage seen in the microwaved *Scenedesmus* can be interpreted as biological fact.

Our results indicated that the microwave technique improved extraction yield for all four genera of cultured phytoplankton. The ratio of total chlorophyll

(microwaved/control) was always greater than 1. *Tetraselmis* (a green algae) in older cultures extraction yield of total chlorophyll tends to be lower unless the microwave technique is used. The chlorophyll from the 2.6 month (79 day) old culture at the time of the experiment was extracted almost 3.6x better with the microwave technique (Fig. 14). This indicates that the microwave technique is more effective and maximizes the amount of chlorophyll that is obtained from the *Tetraselmis* culture.

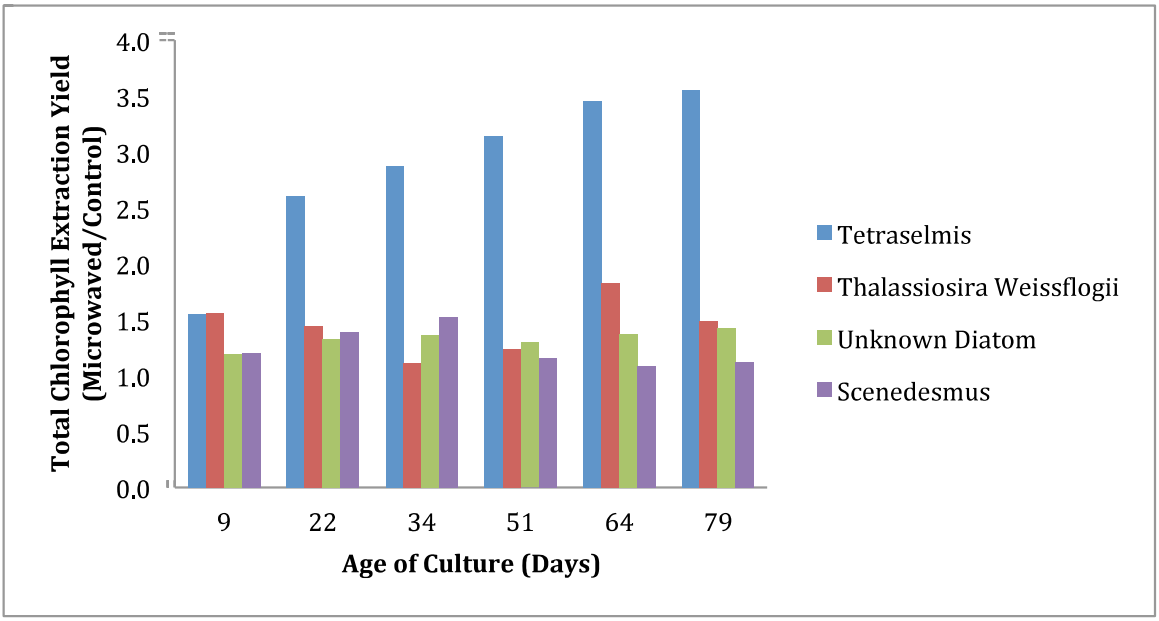


Fig. 14: Chlorophyll extraction yield from 4 different genera of cultured phytoplankton. Yield is expressed as the ratio of microwave treated samples to control soak samples.

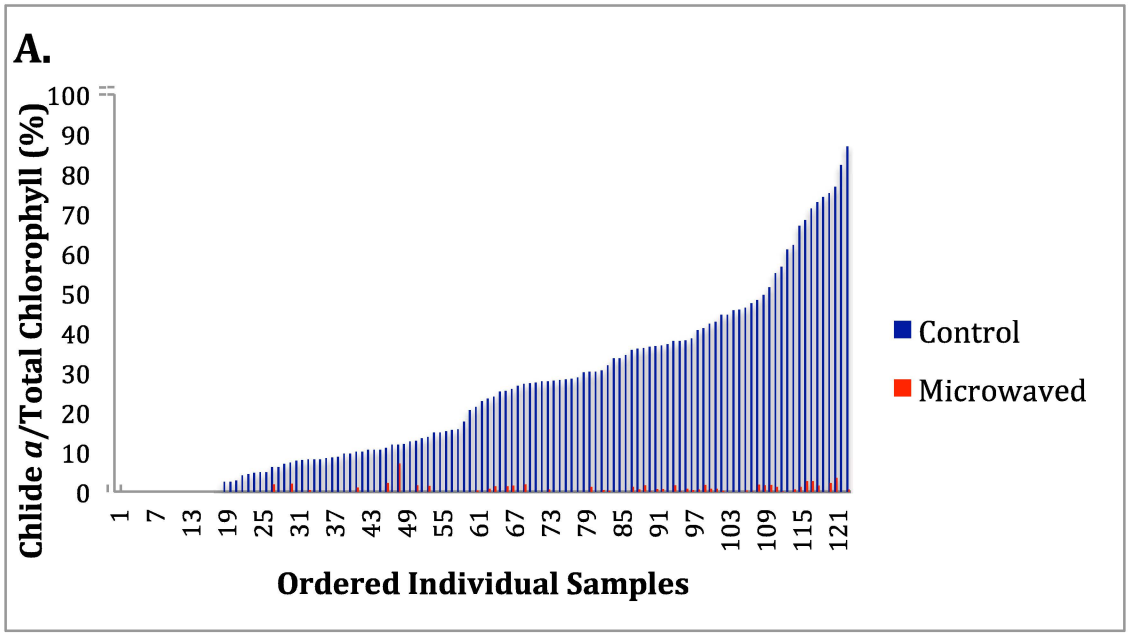
Table 5: Chlorophyllide *a* % in 4 different cultured species.
C= Control, M= Microwaved. Total Chlorophyll (Chl *a* + Chl *a*' + Chlide *a*).
UD=undetected

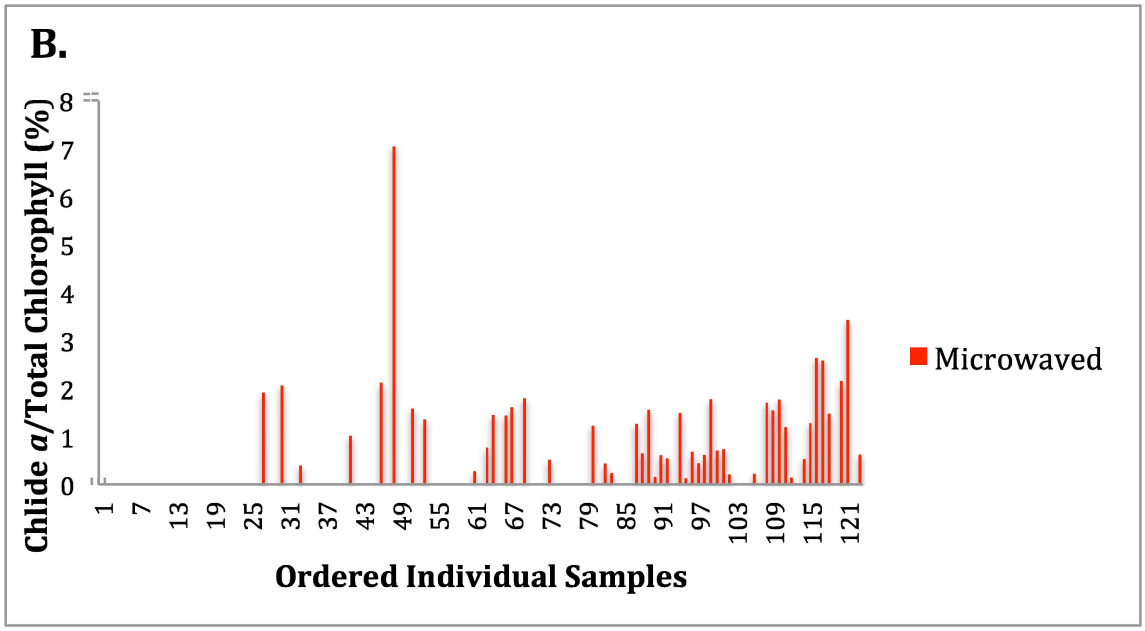
	Tetraselmis Chlide *a* %		*Thalassiosira Weiss* Chlide *a* %		Unknown Diatom Chlide *a* %		*Scenedesmus* Chlide *a* %	
	C	M	C	M	C	M	C	M
1) 9 days old	UD	UD	2.6	UD	UD	UD	7.3176	2.0595
2) 22 days old	UD	UD	2.9	UD	4.1109	UD	11.9058	7.0473
3) 34 days old	UD	UD	5	UD	4.3859	UD	6.2605	1.9142
4) 51 days old	UD	UD	6.3	UD	4.8123	UD	11.9058	2.1211
5) 64 days old	UD	UD	9.6	UD	13.4809	UD	22.7804	UD
6) 79 days old	UD	UD	7.8	UD	10.1477	UD	13.7698	1.3530

Summary of Effect of Microwaving on Chlorophyllide *a* measurements in Environmental and Culture Samples

The collective results from all of the assays reported here are summarized in Fig. 15a, b and showed that the microwave-assisted extraction technique greatly reduced the amount of chlorophyllide *a* formation compared to a solvent soak technique (Fig. 15a). In the control samples, chlorophyllide *a* % ranged from 0 % to 89% (Fig. 15a, blue bars), with approximately half of the 121 total control samples ranging from 20% to 89%. The mean percentage of chlorophyllide *a* for the control samples was 24.75%. When the microwave technique was applied all of the chlorophyllide *a* % levels were below 10% (Fig. 15b). The mean percentage of chlorophyllide *a* in microwaved samples was 0.48%. This indicates that there was only a small amount of chlorophyllide *a* present in the sample prior to extraction, if at all. We conclude that most of the chlorophyllide *a* detected in the natural phytoplankton communities and in cultured phytoplankton is an artifact of the extraction with the common solvent soak technique (Fig. 15a, blue bars). The microwave assisted extraction protocol inhibits chlorophyllase enzyme activity so the artifactual production of chlorophyllide *a* was greatly reduced; the residual low levels of chlorophyllide *a* after microwave treatment likely represent best estimates of the true *in situ* cellular levels (Fig. 15b). The microwave-assisted

extraction also significantly increased pigment extraction yield giving best estimates of actual total chlorophyll. A summary plot of the ratio of microwave/control extraction methodology from all samples reported here is given in Fig. 15c (n=121 paired samples). Ninety eight percent of the samples had a better extraction yield when the microwave technique was used, with an average increase of 2x (Fig. 15c). Our results indicate that the solvent soak technique as currently used could lead to an underestimation of total chlorophyll up to 4x.





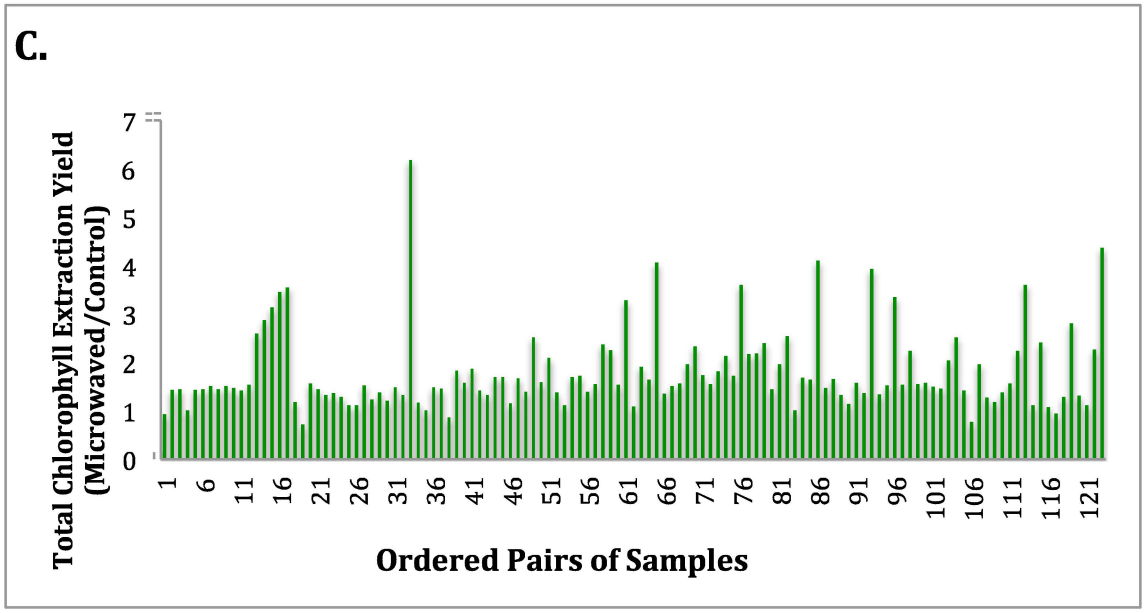


Fig. 15: Chlorophyll *a* and Chlorophyllide *a* in 121 samples. A) Chlorophyllide *a* % in 121 samples. Control (blue) samples have matched microwaved (red) samples were extracted using optimized conditions (90% acetone, 20 seconds of microwaving for microwaved samples). The mean percentage of chlorophyllide a in the control was 24.75 and microwaved the mean percentage decreases to 0.48%. **B)** Detail of remaining chlorophyllide *a* % with microwaving across all samples. **C)** Total Chlorophyll (Microwaved/Control) across all 121 individual samples. Samples here were ordered by increasing chlorophyllide a % (same as panel A and B).

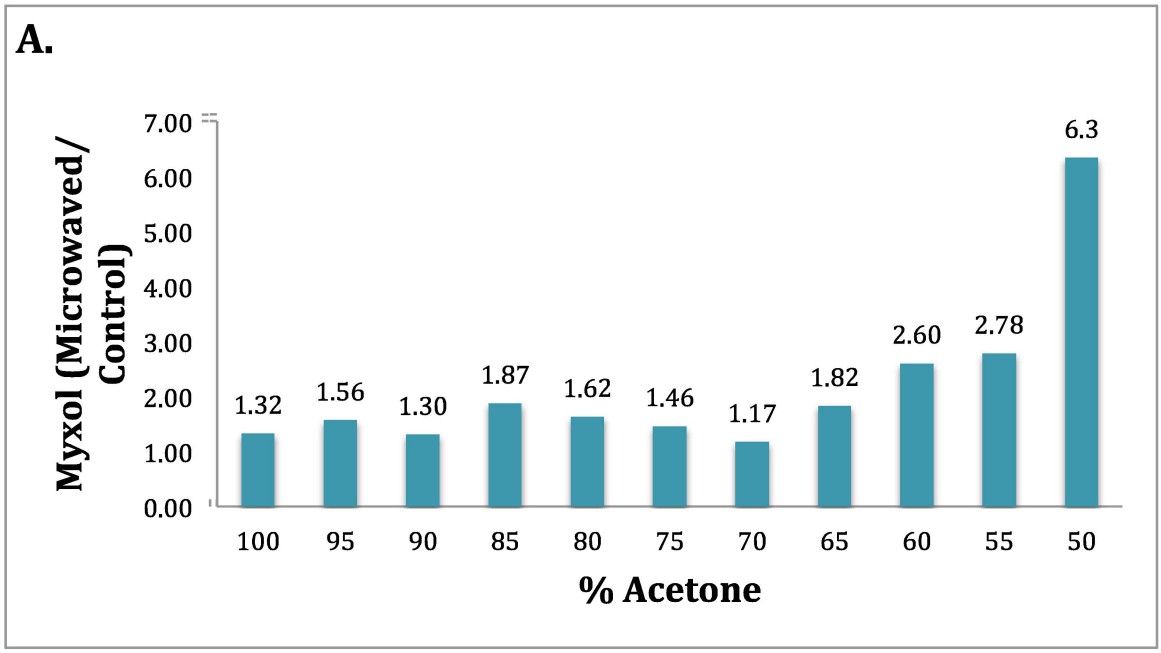
Application of the Microwave Technique: Effect on Carotenoids

A partial analysis of the effects of microwave-assisted extraction methodology is given here for some of the more common lipid soluble carotenoids. The reported data considers the potential increase in extracted carotenoid yield. Carotenoids such as myxol showed a similar increase in extraction yield (Microwaved/Control) as chlorophyll *a*-like pigments (Fig.16a,b). Most carotenoids showed better extraction when the microwave technique was applied. For all of the carotenoids measured in the Lake El Estero water samples there was an improved extraction yield with the application of the microwave technique. Different carotenoids emerge as more dominant than others in different environments depending upon the composition of the phytoplankton community. In particular, in the Lake El Estero sample Myxol was one of the most dominant carotenoids. Myxol is a carotenoid that is associated with the photosynthetic apparatus of cyanobacteria. Extraction yield of myxol increased in all the microwaved samples independent of acetone dilution or extraction temperature. In 50% acetone the extraction yield was so poor in the non-microwaved control sample that the microwave technique improved it by 6x (Fig. 16a). Myxol also showed a notable increase in the Freezer sample and Freezer combined with Liquid Nitrogen (20 secs) treatment (3x better) when the microwave technique was applied (Fig. 16b). Myxol extraction yield increased in all extraction temperatures when the microwave technique was applied.

Another pigment that increased with the microwave technique was beta carotene, a compound that lies on the biosynthetic pathway for most other carotenoids in microalgae and higher land plants (Bogacz-Radomska and Harasym, 2018). Beta carotene is made up of a long 40 carbon chain with cyclic rings at either end (Fig. 17). The microwave technique increased the extraction yield for beta carotene in all dilutions of acetone (Fig. 18a). The microwave technique also improved beta carotene extraction yield in different extraction temperatures. The application of the microwave technique improved beta carotene extraction yield by 4x for samples stored in the freezer (Fig. 18b). In the freezer with an added liquid

nitrogen treatment (20 secs) the extraction yield was 5x better after application of the microwave technique. There was also an increase in beta carotene in all four different temperature treatments in the microwaved samples (Fig. 18b).

The microwave technique improved extraction yield for most carotenoids in the cultured samples as well. Beta carotene is better extracted with the microwave technique by up to 2x and in some cases even 3x. With the application of the microwave technique, beta carotene extraction yield increased in all 4 cultured phytoplankton examined. In the green alga *Tetraselmis* where chlorophyll extraction was more difficult in the older cultures beta carotene followed a similar pattern (Fig. 18c). The increase of extraction yield of the microwave technique was widespread amongst all chlorophylls and carotenoids observed.



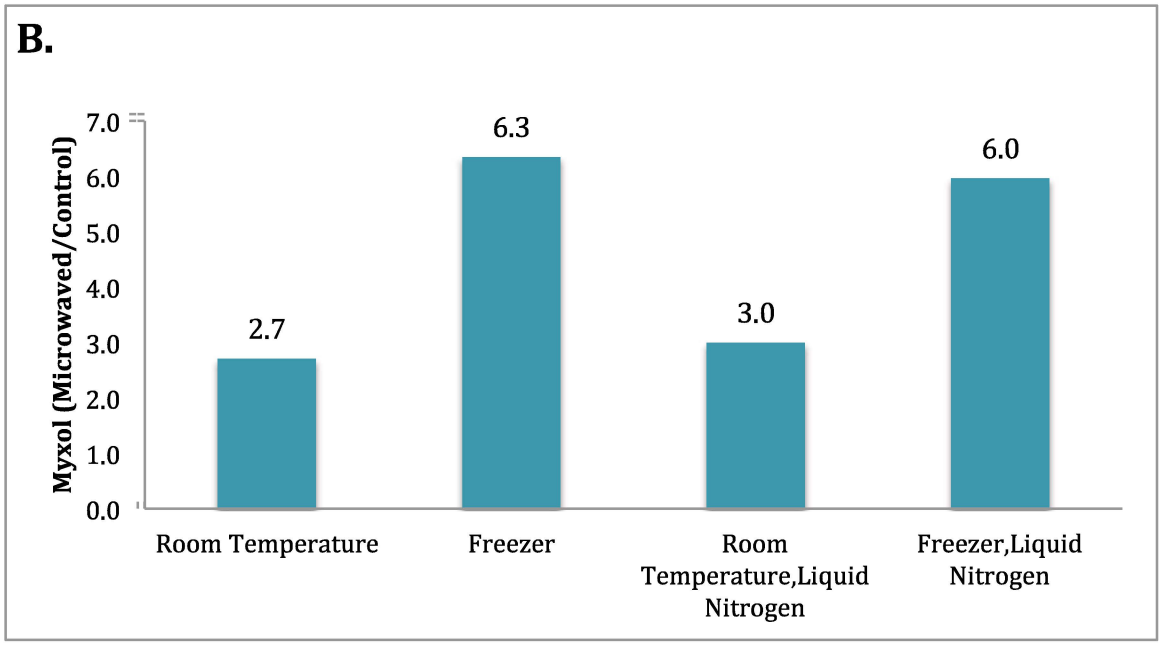


Fig. 16: Effect of microwaving on myxol extraction. A) Percent of acetone effect on Myxol extraction yield ratio. Myxol extraction yield (microwaved/control) is increased when the microwave technique is applied in all acetone dilutions investigated. **B)** Myxol extraction yield (microwaved/control) ratio in different extraction temperatures in Lake El Estero water.

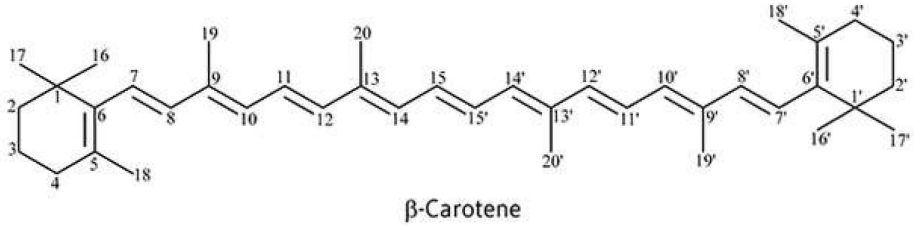
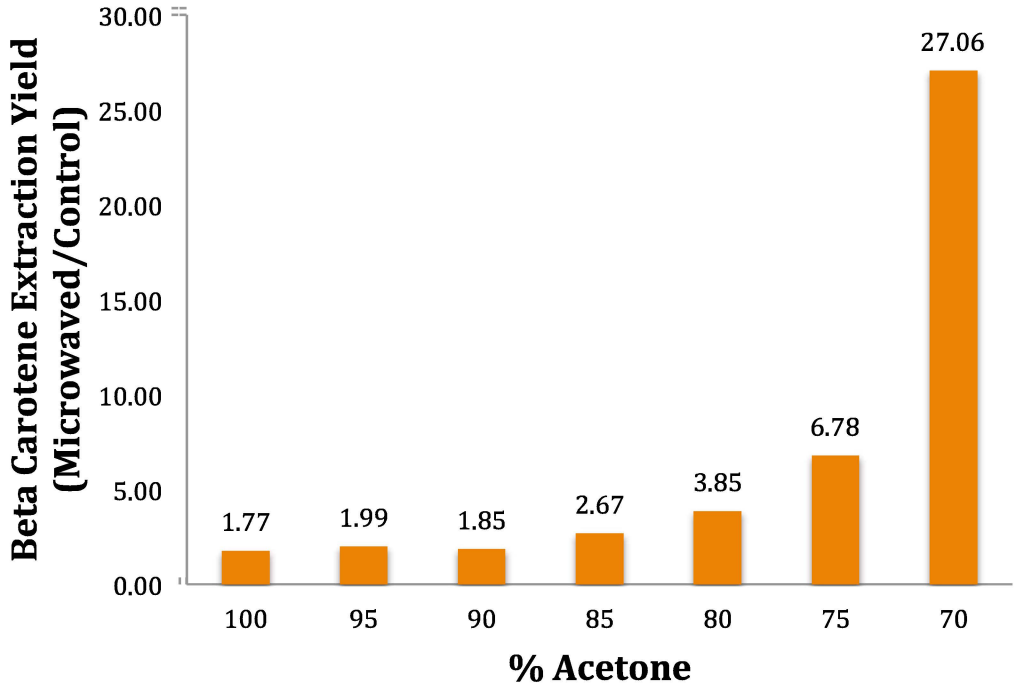
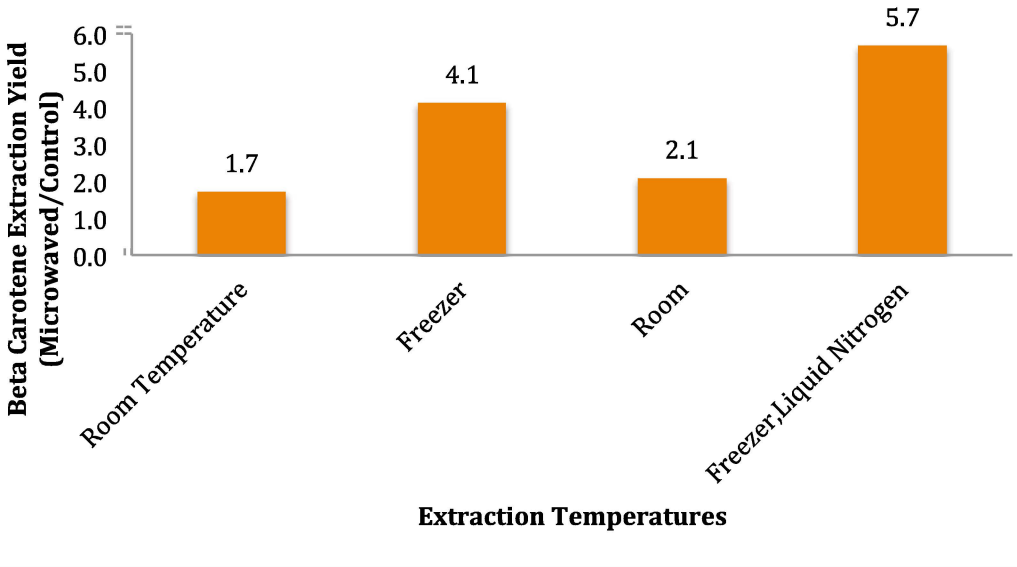


Fig. 17: Structure of beta carotene. Adapted from Allen and Williams 1998.

A.


Beta Carotene Extraction Yield (Microwaved/Control)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
1.77
1.99
1.85
2.67
3.85
6.78
27.06
100
95
90
85
80
75
70
% Acetone

B.


Beta Carotene Extraction Yield (Microwaved/Control)
6.0
5.0
4.0
3.0
2.0
1.0
0.0
1.7
4.1
2.1
5.7
Room Temperature
Freezer
Room
Freezer,Liquid Nitrogen
Extraction Temperatures

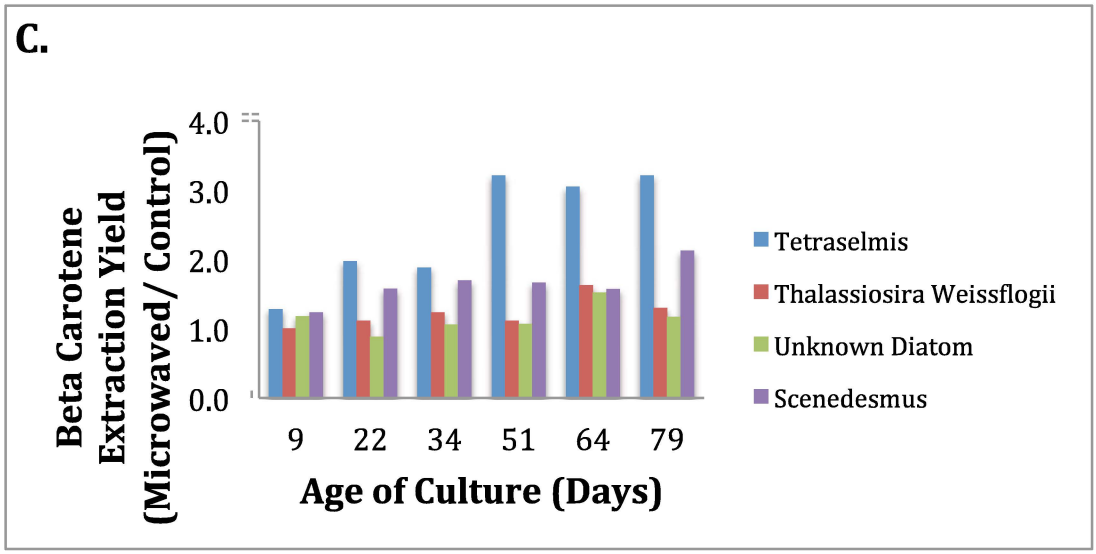


Fig. 18: Effect of microwaving on beta carotene extraction.
A) Percent of acetone effect on beta carotene extraction yield.
B) Beta carotene Extraction yield (microwave/control) in different extraction temperatures. **C)** Beta carotene extraction yield (microwaved/control) in 4 different genera of cultured phytoplankton.

Discussion

Phytoplankton communities serve as large sinks of carbon; most models of primary production utilize bulk chlorophyll *a* as the normalizing autotrophic biomass indicator. Therefore, accurate assessment of the true levels of chlorophyll in the ocean is important. Numerous methods have been proposed such as sonication, mechanical grinding and solvent wet-soak in an effort to obtain the highest yield of chlorophyll extraction (and thus, the most accurate measure of in-*situ* chlorophyll concentrations). However, these methods are limited in that yield can vary between pigments and between samples and that artifactual formation of chlorophyllide *a* can reduce estimations of chlorophyll when pigments are separated by analytical chromatographic methods.

Here, we determined that microwaving of plankton samples harvested on filters prior to solvent extraction yields two desirable improvements to the routine determination of chlorophyll *a*: 1) an increase in total chlorophyll extraction yield and 2) reduction of the artifactual production of chlorophyllide *a*. The HPLC instrument was used in this study because it can separate chlorophyll *a* from its breakdown product chlorophyllide *a*. Hu et. al (2013) argued that a drawback of the HPLC technique (relative to the bulk fluorometric/absorbance methods) was the erroneous production of artifactual chlorophyllide *a* during the pigment extraction process, leading to questionable estimates of true chlorophyll *a* concentration. Our results confirm that chlorophyllide *a* is an artifact of the routine chlorophyll acetone soak extraction method, and is likely a probable error in all extraction technologies that do not provide a means of enzyme deactivation. Our results suggest that the chlorophyllase enzyme activity is greatly reduced by microwave treatment. An initial concern, that microwave treatment (e.g. heating) may cause bulk destruction of many pigments, was found not to be the case. In contrast, microwave assisted extraction resulted in higher yields of total chlorophyll *a*-like pigments in 96% of all cases tested, with an average of a 2x increase in total chlorophyll *a*, relative to the common chilled acetone soak extraction. Since chlorophyllide *a* formation is no longer occurring, the microwave technique combined with HPLC has eliminated a decades old problem for chlorophyll analysis. The optimized microwave chlorophyll

extraction technique described here is simple, cost-effective and will likely lead to more accurate assessments of chlorophyll and other pigments in the ocean. This technique is also directly applicable to simple bulk chlorophyll measurements using absorbance and/or fluorescence.

A major focus of this study was to optimize this method for pigment analysis. The presence of water in the extraction solvent affects the amount of chlorophyllide *a* artifactually produced. However, with microwaving the formation of chlorophyllide *a* is minimized across a wide range of acetone: water ratios and therefore this problem is almost completely eliminated. Chlorophyllide *a* was greatly reduced after filters were microwaved in a conventional microwave (1500 W full power) for 20-30 seconds. This technique was applied to cell cultures to evaluate suggestions that chlorophyllide *a* is formed in cells during stress induced nutrient limitation (Hallegraeff 1980). Microwave-assisted extraction did show chlorophyllide *a* concentrations in many natural environmental samples and nutrient deprived algal batch cultures, but chlorophyllide *a* concentrations were extremely low (generally undetectable or <2% of total chlorophyll *a* like pigments. Therefore, it is suggested that previous observations of high chlorophyllide *a* content in phytoplankton samples are simply an artifact of the extraction process.

We compared different extraction temperatures to further challenge the microwave technique. In all extraction temperatures, the microwave technique increases total chlorophyll extraction yield when compared to the routine chlorophyll analysis. An oceanographer or limnologist looking for consistency in their samples, independent of how samples are stored, should consider the microwave technique as optimal. This study showed that the HPLC analysis combined with the microwave technique can be utilized to understand chlorophyllide *a* concentrations in nature without the uncertainty of artifactual production of chlorophyllide *a* due to the extraction process.

The microwave technique applied to chlorophyll analysis can improve extraction of pigments and can provide us with more accurate estimates of primary production in our world's oceans. Other pigments such as carotenoids also show improved extraction yield with the application of the microwave technique. For

example, beta carotene showed higher extraction yield, often in excess of a factor of two. We showed that beta carotene, a major carotenoid present in most microalgae, is harder to extract than any other dominant pigments observed. Myxol also showed increased extraction yield with the application of the microwave technique.

We expect that this improved pigment analysis method will be important beyond the field of biological oceanography, specifically in higher land plant physiology and the study of kelp and freshwater algae. Importantly, our results also show that there could be an underestimation of global oceanic primary production in all chlorophyll-irradiance models of satellite color imagery that have been calibrated using common acetone soak extraction procedures.

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