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Optimization and characterization of the bulk FDA viability assay to quantify living planktonic biomass

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OPTIMIZATION AND CHARACTERIZATION OF THE BULK FDA VIABILITY ASSAY TO QUANTIFY LIVING PLANKTONIC BIOMASS

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OPTIMIZATION AND CHARACTERIZATION OF THE BULK FDA VIABILITY ASSAY TO QUANTIFY LIVING PLANKTONIC BIOMASS

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ABSTRACT

Optimization and characterization of the Bulk FDA viability assay to quantify living planktonic biomass

by
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Master of Science in Marine Science
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The detection and quantification of viable aquatic biomass, especially of the microbial community, is a fundamental aspect of ecological, oceanographic, environmental and other specialized fields. The abundance and activity of aquatic microbial communities and how they change in space, time or in response to some environmental perturbation are subjects of significant research interest. Environmental management officials and technicians must quantify viable marine microorganisms in waste, gray, drinking and ballast water to determine if regulations are met. Unfortunately, few methods exist to assess viable biomass; those that do are often laborious, unreliable, expensive, qualitative rather than quantitative, or restricted to the measurement of a specific group of organisms. Moreover, the distinction between living and dead can be ambiguous for marine microorganisms, whether this distinction is made visually or via an indirect process, such as a chemical indicator. A need exists in scientific and public sectors to develop a convenient means to quantify the total, or bulk, viable biomass present in any water sample. A method for determination of total living biomass based on bulk fluorometric detection in simple optical reaction cuvettes has recently been developed (Welschmeyer and Maurer, 2013); the method, termed the Bulk FDA technique, is based on quantitative conversion of fluorescein diacetate (FDA) to the fluorescent product, fluorescein, by ubiquitous enzymes in living organisms. This thesis describes the optimization and characterization of the Bulk FDA technique. The optimized assay should prove useful in a wide range of academic and regulatory applications.

TABLE OF CONTENTS

PAGE

LIST OF TABLES

TABLE OF FIGURES

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INTRODUCTION

The Invasive Species Problem

Aquatic invasive species are a significant threat to the well-being of global ecosystems. Aquatic invasive species are organisms and plants which are introduced into new environments. Once introduced, non-native species can become established, threatening the well-being of native ecosystems and the resources within them. Invasive species can out-compete native organisms for resources or habitat, resulting in the decline or elimination of native species. The majority of invasions result from the transport of organisms from one place to another by human activity, and the most significant transport mechanism, or vector, contributing to aquatic biological invasions is the uptake, transport and subsequent dumping of ballast water by shipping vessels (Ruiz et al., 1997), although it is now recognized that hull-fouling represents a significant source of invasions as well (Drake et al., 2007). Presently, the rate and impact of biological invasions is increasing; as a result, increasing attention is being given towards the management of aquatic biological invasions (Ruiz and Carlton, 2002).

The International Maritime Organization, United States Coast Guard, and other international agencies have recently imposed regulations requiring sterilization of water to be de-ballasted. These regulations provide specific and quite stringent requirements regarding the numeric concentration of viable organisms permitted in three distinct size classes (<10µm, 10-50µm, >50µm). Methodology does not currently exist which is sensitive, reliable and efficient enough to routinely evaluate ballast water treatment system performance at the low living biomass levels required by regulations. This research sought to improve the existing Bulk FDA technique, making it sensitive, reliable and efficient such that is a useful tool for evaluating ballast water treatment system efficacy, as well as other oceanographic parameters.

The Quantification of Viable Biomass

The measurement of viable aquatic biomass is often required in ecological research to provide insight into transfer and cycling of energy and materials through the

microbial community, including zooplankton, phytoplankton, bacteria and viruses. The size distribution of living particles in aquatic environments is a subject of longstanding research interest (Sheldon et al., 1972) ; measurements of bulk viable biomass among discrete size ranges can provide insight on this topic (Rinaldo et al., 2002). Estimates of viable biomass are often more relevant than live cell concentration in ecological research (Holm-Hansen 1970), as metabolic activity, and therefore the effect that a group of organisms will have on their environment, scales with biomass rather than numeric concentration. Measurements of viable biomass can also assist in investigating the distribution and abundance of viable organisms in space or time, such as the response of a microbial community to environmental perturbation (Steward et al., 1996). Recently, the measurement of viable biomass has become necessary to evaluate the performance of ballast water treatment systems.

Current Methods to Measure Viable Biomass

A variety of methods currently exist to measure viable aquatic biomass, each with its own advantages and disadvantages.

ADENOSINE 5'TRIPHOSPHATE (ATP)

Microbial biomass can be estimated by the extraction and measurement of adenosine 5'triphosphate (ATP), the energy currency for living cells. Numerous publications have investigated the relationship between ATP and living biomass in aquatic environments over the past 40 years (Hamilton and Holm-Hansen 1967, Karl 1993). The extraction of ATP is relatively simple in a laboratory setting, requiring cells to be concentrated and boiled in aqueous buffer (Tris) for a short time, then frozen until analysis. The quantitative determination of extracted ATP is problematic, especially in the field, as it depends on the activity of a rapidly degrading enzyme, luciferase, and requires specialized instrumentation (luminometer) and at least 1 empirical calibration each time samples are run. The ATP assay is prone to the "filtration effect" (Karl and Holm-Hansen 1978), in which desiccation immediately after filtration results in a rapid

degradation of cellular ATP. Also, because of the ubiquitous nature of ATP, the assay is prone to contamination.

CHLOROPHYLL *A*

All oxygenic phytoplankton contain chlorophyll *a* (chl *a*), the primary pigment involved in photosynthesis. Because chlorophyll *a* is fluorescent, it can be quantified by spectrofluorometry, either extracted in an organic solvent for the most precise measurement, or with field *in situ* or portable fluorometers. Chl *a* concentration per unit phytoplankton biomass is relatively constant. Chl *a* is not, however, a reliable indicator of viable algal biomass, as it remains intact and fluorescent in dead cells for extended periods of time. Also, because chl *a* is only present photosynthetic organisms, heterotrophic biomass must be quantified by some other means.

PHOTOSYNTHETIC ^{14}C INCORPORATION

The photosynthetic activity of phytoplankton can be quantified by measuring the incorporation of inorganic ^{14}C labeled bicarbonate into phytoplankton biomass after an incubation period. Viable phytoplankton will fix the dissolved radioactive inorganic carbon into their tissue as particulate organic carbon through photosynthesis; dead cells will not fix radioactive carbon. The rate of ^{14}C incorporation can be used to estimate the quantity and/or activity of photosynthetic organisms, but does not measure heterotrophic growth.

PULSE AMPLITUDE MODULATED (PAM) FLUOROMETRY

PAM fluorometry is used to measure variable fluorescence, an indicator of algal cell physiological health. Variable fluorescence indicates the presence of algal cells that are capable of using incident light for photosynthesis, as well as other processes. High levels of variable fluorescence indicates healthy, viable cells. While both the ^{14}C and PAM techniques detect the presence of viable algal cells, neither is ideal for estimating numeric abundance of viable algal cells and both are limited to the detection of autotrophic cells.

MICROSCOPE COUNTS

Regulations governing treated water are commonly written in the units of numeric concentration of living cells allowed in distinct size classes, making numeric quantification of living cells necessary. One of the most commonly applied techniques to quantify the numeric concentration of live organisms is by direct microscopic count. Large and motile organisms, such as zooplankton, can be counted microscopically using the “poke and probe” technique to determine if organisms are alive or dead. An organism displaying organ activity or motion on its own or in response to a "poke" is presumed alive, while an organism that does not respond when probed is presumed dead. This technique cannot, however, make the distinction between live and dead for organisms which are non-motile, such as many phytoplankton species. In order to make a direct live count of non-motile organisms, a marker or stain that distinguishes live and dead cells must be used. Many viability markers are commercially available, most of which are colorless molecules that become fluorescent and detectable in response to an indicator of viability, such as enzyme activity (fluorescein diacetate (FDA), cmFDA, calcein AM). Other stains are used to identify dead cells, such as Sytox Green, a DNA stain that marks cells with compromised plasma membranes. Analysis using these stains and markers is often challenging. Viability markers, especially FDA, leak out of cells over time and the amount of leakage varies by cell type. This leakage creates a time constraint on sample analysis. Variable fluorescence levels as fluorescein leaks can make the distinction between live and dead cells ambiguous. False positives can be a problem with fluorogenic viability indicators, such as actively swimming dinoflagellates tagged as "dead" by Sytox green (Prince et al., 2008). Microscopic direct count techniques are tedious, prone to user error and statistically challenged due to the low volumes of sample analyzed.

FLOW CYTOMETRIC COUNTS

Flow cytometry functions by focusing particles and/or cells into a single file stream and measuring the fluorescence and scattering characteristics of individual particles. Live cells can be enumerated by employing the same stains and markers as used in epifluorescence microscopy; an increase in intracellular fluorescence indicative of

metabolic activity or the presence of DNA is readily detected by the instrument. Flow cytometry counts improve upon epifluorescent counts by eliminating the human interpretation of fluorescence and size. However, the same issues of marker leakage, false positives and low analysis volumes that plague microscope count techniques are present in flow cytometric analysis. Flow cytometric analysis is also restricted to cells <70 micron, as the aperture is easily clogged by large particles.

GROW OUT TECHNIQUES

Grow out methods are among the most trusted and commonly used methods to quantify or identify the presence of live organisms; a sample that exhibits growth clearly possesses live cells or organisms. Live bacteria and phytoplankton can be quantified by Most Probable Number (MPN) methods in which serial dilutions of sample water are made in many replicates and scored for growth after an incubation period. The change in bulk Chl *a* fluorescence over time provides the assessment of algal cell growth, while colony formation indicates bacterial growth. An algorithm calculates the most probable initial concentration of live cells based on the number of replicates showing growth at each dilution. Heterotrophic plate counts (HPC) are used to quantify cultivable bacteria; sample water is spread in a thin layer on agar plates and scored for colony formation after an incubation period. Unfortunately, most aquatic microorganisms from natural samples do not grow well in a laboratory setting (Staley and Konopka 1985, Connon and Giovannoni 2002). It is estimated that only 1% of marine bacteria, for example, are cultivated with the HPC technique (Wagner et al., 1993). Thus, grow-out techniques are poorly-suited for quantitative determination of live cell concentration; estimates of live cell concentration by grow-out techniques are inherently conservative.

Esterases and Fluorescein diacetate

Esterases are cytoplasmic enzymes that are present in all domains of life (Rotman and Papermaster, 1966) and function by hydrolyzing esters into alcohol and acid constituents. There are many different types of esterases that function in a wide range of biological processes. These include lipases, which cleave lipids, and acetylchloinesterases, which hydrolyze the neurotransmitter acetylcholine. In algal cells,

esterases are essential for the turnover of phospholipids in membranes, which is correlated with metabolic activity (Dorsey et al., 1989). Measurements of esterase activity (EA) can be used to assess if an organism, or group of organisms, is alive or dead (Steward et al., 1999). Esterases are present in all marine organisms, and are only present in live or recently deceased organisms. As such, they make an excellent proxy for bulk viable biomass.

Fluorescein diacetate (FDA) has been routinely applied to assess cell-specific metabolism and viability in single and multi-cellular organisms (Green et al., 2006). FDA was introduced almost 50 years ago as a chromogenic viable cell marker (Rotman and Papermaster, 1966), making it one of the oldest viability detection methods in use today. FDA is a non-fluorescent, non-polar, esterified compound that freely enters both live and dead cells. Inside a metabolically active cell, FDA is hydrolyzed by cytoplasmic esterases, lipases and proteases, which remove the 2 acetate groups and produce the green fluorescent, polar compound fluorescein. The rate of fluorescein production by a cell reflects its cumulative EA. The polar nature of fluorescein causes it to initially accumulate inside cells, enabling the detection of bright green fluorescent viable organisms by epifluorescence microscopy or flow cytometry. However, over time, fluorescein will leak out of cells and into the extracellular medium (Rotman and Papermaster, 1966, Jochem, 1999).

The green fluorescent molecule fluorescein was first prepared by Adolf von Baeyer in 1871 by fusing a mixture of pthalic anhydride and resorcinol. In 1927, fluorescein diacetate (FDA), a colorless derivative of fluorescein, was created by Orndorff and Hemmer. In 1966, Rotman and Papermaster used FDA to describe the pattern and kinetics of intracellular fluorescein accumulation and leakage in cultured mammalian cells, showing that intracellular fluorescein accumulation occurs only in viable cells with intact cell membranes. The vast majority of studies involving FDA have used the marker to indicate cell-specific viability, however, several efforts have been made to use FDA as a bulk indicator of living biomass. Swisher and Carroll (1980) demonstrated that FDA hydrolysis correlated with cumulative biomass of fungi, bacteria and algae growing on Douglas fir foliage. Schnurer et al. (1982) developed a protocol using FDA to measure the activity of microbes in soil and litter, and found that FDA

hydrolysis rates increased linearly with the addition of soil and bacteria cultures, and was correlated with respiration rates in soils. These efforts to measure bulk viable biomass with FDA were complicated by high non-biological fluorescein production in the incubation solutions without live cells (Clarke et al., 2001), resulting in large "blanks" and associated decreases in sensitivity. Because the density of living biomass is quite high in environments such as soils, these bulk assays involving FDA are useful despite significant non-biological fluorescein production. Aquatic environments, in contrast, typically show much lower density of living organisms. High non-biological fluorescein production in the incubation solutions renders these assays too insensitive to be useful in aquatic environments.

The Bulk FDA technique

The Bulk FDA technique, which takes advantage of fluorescein leakage to achieve a bulk indication of esterase activity from a heterogeneous ballast water sample, was first described for ballast water work by Welschmeyer and Maurer (2011). Recently, the method has been described for general use in determination of living biomass for a broad spectrum of microbial research areas (Welschmeyer and Maurer, 2013); this publication presents the fundamental aspects of the technique in detail. Some of the data presented therein are summarized below to provide the starting point for the optimization process.

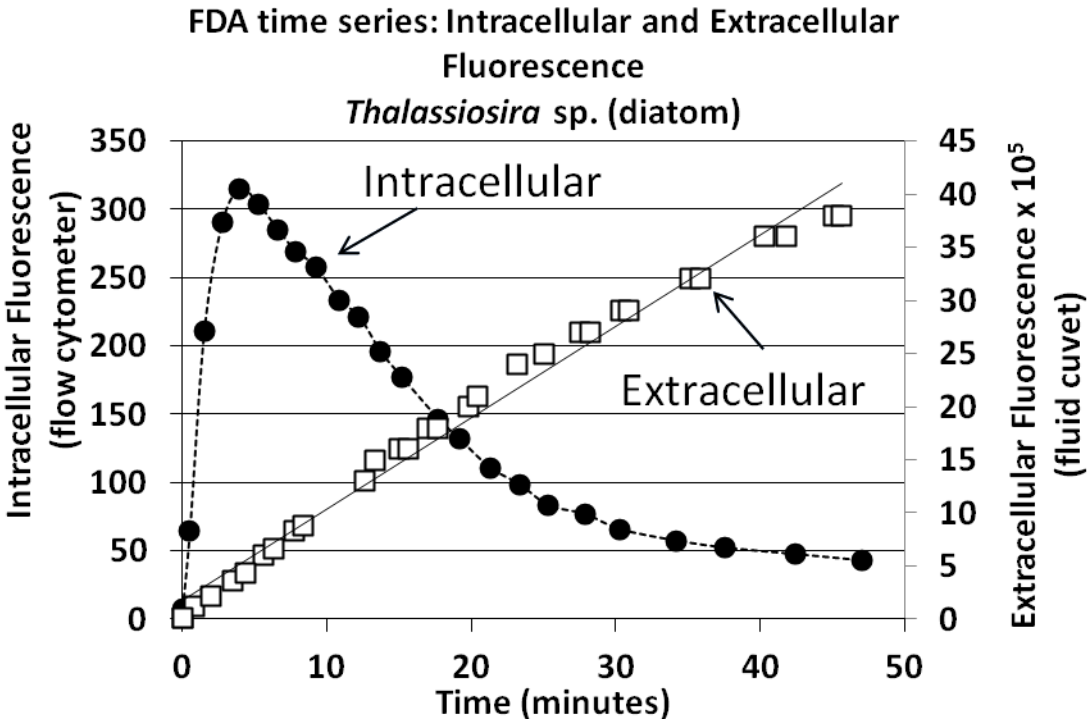
CHARACTERIZATION OF FLUORESCEIN LEAKAGE

Intracellular fluorescein accumulation and subsequent leakage out of cells which occur during an incubation with FDA was characterized quantitatively using several algal cultures. The general pattern was essentially the same for all cell types tested, varying in the quantity of fluorescein accumulated and leaked and the duration of each phase (Figure 1). Upon addition of FDA, intracellular fluorescence rises rapidly to some maximum level, then begins to decrease as fluorescein leaks out of cells. The cumulative leakage of all cells being assayed results in a linear increase of extracellular fluorescein concentration over time. Extracellular fluorescein production (FP) forms the basis of the Bulk FDA technique.

FLUORESCEIN PRODUCTION IS PROPORTIONAL TO LIVING BIOMASS

The relationship between FP and biomass filtered was demonstrated by quantitatively filtering a range of volumes of algal cultures and size fractionated natural samples onto GF/F and 10μm nylon filters, creating gradients of biomass loaded, and carrying out incubations with FDA. Linear relationships were observed between biomass

Figure 1: The pattern of intracellular fluorescein accumulation and leakage during an incubation with FDA and the pattern of extracellular fluorescein production (FP) during the same incubation



and FP, confirming that the Bulk FDA assay responds proportionally to biomass filtered (Figure 2).

Cultured *Dunaliella* sp. cells treated with a variety of known kill factors show reduced or eliminated FP relative to untreated controls (Figure 3) (all control values were normalized to 1). Ballast water samples were collected aboard the T.S. Golden Bear in Kobe, Japan, Guam and Saipan in June 2010 and in San Francisco Bay in December, 2010; uptake, control and treatment (electrolytic chlorination) ballast water samples were size fractionated and tested with the Bulk FDA technique (Figure 4). Treated samples

show significant reduction of FP in all size classes; corroborative assays showed a similar treatment effect. Size fractionated Bulk FDA data also show the expected trend of biomass distribution among size classes: approximately 70% of the total biomass in uptake samples was present in the smallest size fraction (0.7-10 µm).

Figure 2: Bulk FDA response to biomass gradients, size fractionated Moss Landing harbor water and *Thalassiosira* sp. culture

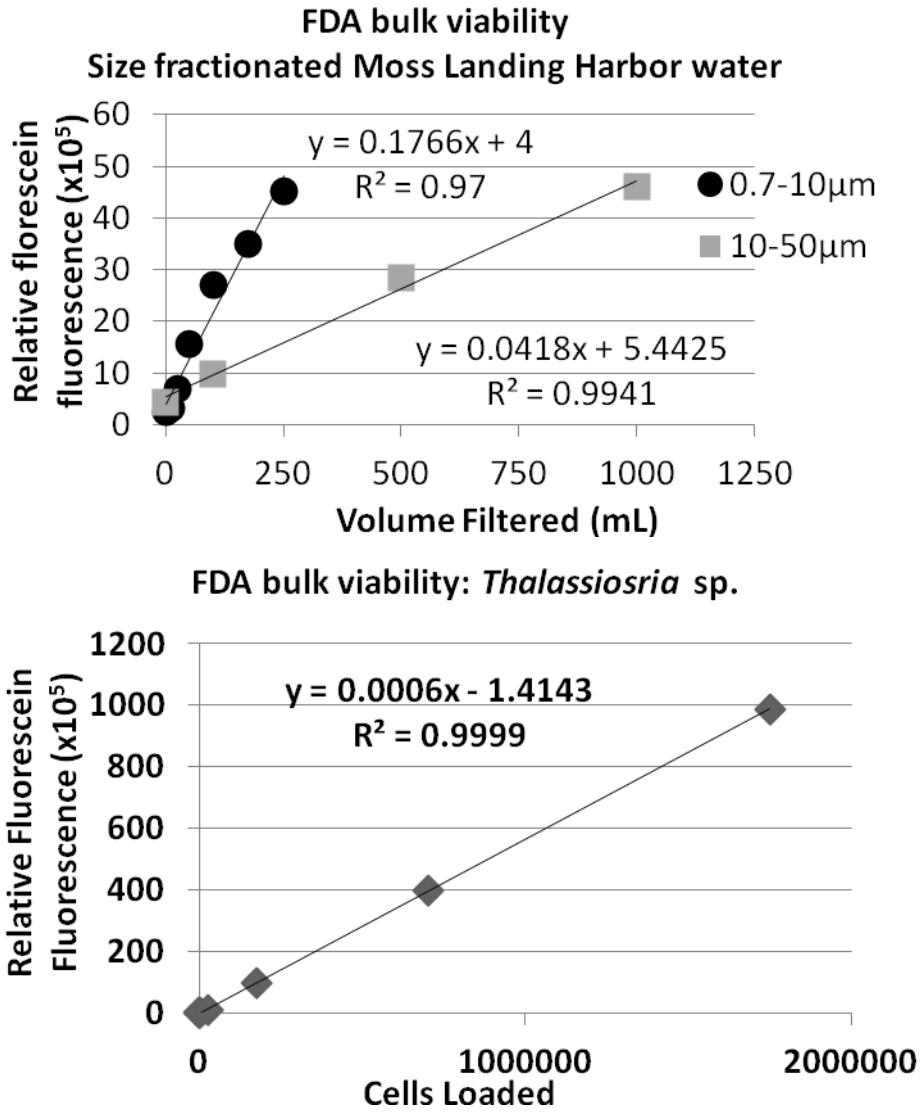


Figure 3: Normalized Bulk FDA response to kill treatments (*Dunaliella* sp.)

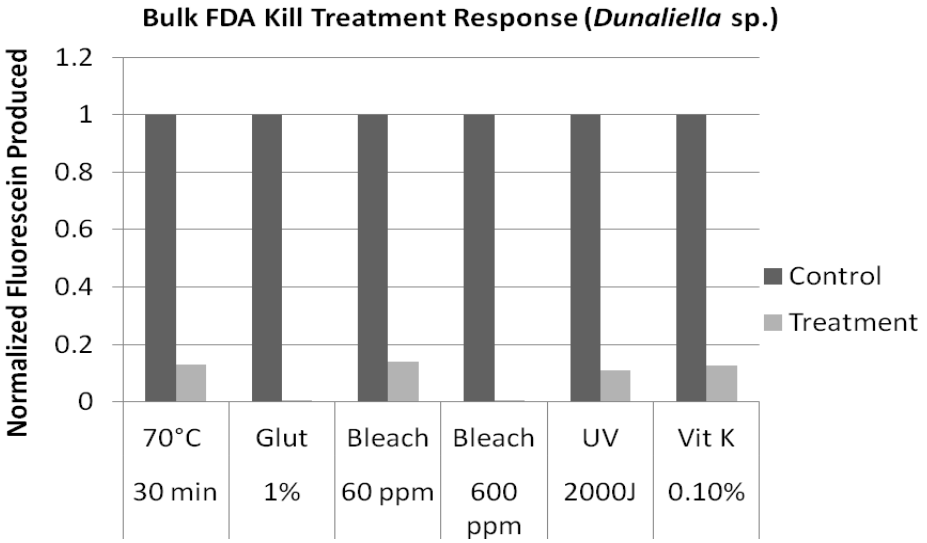
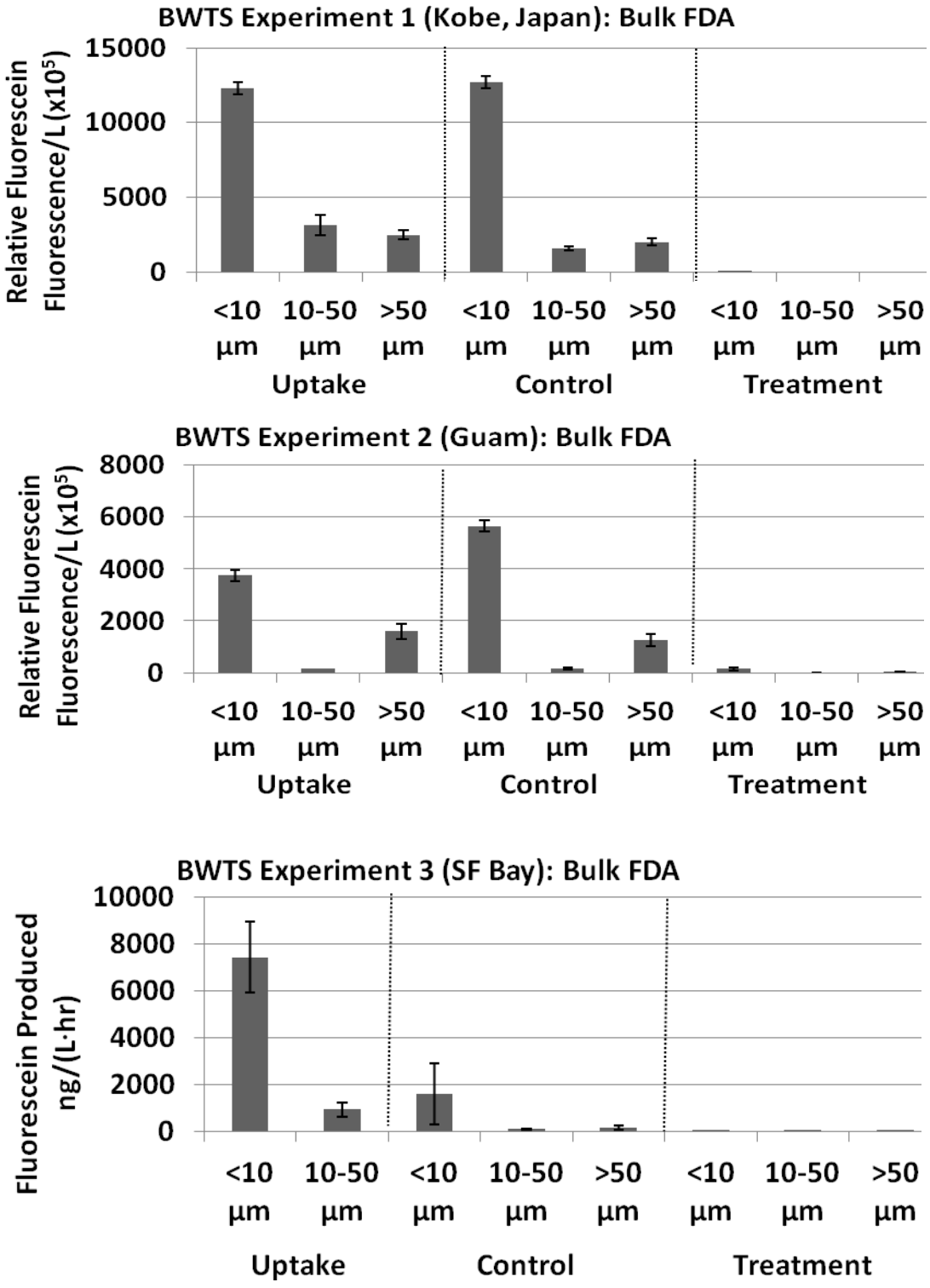


Figure 4: Size-fractionated Bulk FDA response to electrolytic chlorinating ballast water treatment system (BWTS)



THE RELATIVE PROPORTION OF INTRACELLULAR AND EXTRACELLULAR FLUORESCENCE

The Bulk FDA assay depends upon the measurement of fluorescein in the extracellular fluid. The relative proportion of extracellular and intracellular fluorescein that combine to make up total fluorescein was quantified. Equivalent numbers of cultured *Tetraselmis sp.* cells were incubated in the presence of FDA for 1-hour in replicates. Some replicates were measured without removing cells, thus making a measurement of total fluorescein (intracellular + extracellular). Other replicates were measured after removing the cells by filtration, thus removing the intracellular fraction of total fluorescein. On average, extracellular fluorescein makes up 97% of total fluorescein; individual values ranged from 94-99% extracellular fluorescein. Because the majority of fluorescein produced by live cells has leaked out of cells by the end of a 1-hour incubation, cells can be removed prior to making the fluorescence measurement while retaining the majority of the fluorescent signal.

THESIS GOALS

The goals of the current thesis are as follows:

1) Optimize the Bulk FDA technique.

2) Characterize the Bulk FDA technique, including field verification and correlation with other established indicators of living biomass.

METHODS

The Bulk FDA technique

Experiments were preformed over the course of the optimization process; many of the experiments presented are testing aspects of the protocol itself. Therefore, the protocol for the Bulk FDA assay is not identical for all data presented. Moreover, the Bulk FDA technique can be performed in several ways. If more signal is desired, the temperature can be increased or the incubation can run for longer times. Acetone can be added at 1:1 concentration to terminate the reaction if a fluorometer is not available. The final optimized technique is described below. Experiments were carried out according to the following technique, unless otherwise stated.

REAGENTS

Fluorescein diacetate (Sigma-aldrich, part # F7378 MW 416.38): 2.5 mM FDA in DMSO (1.04 mg/mL).

Reagent A: 500mM d-Sorbitol + 20mM MES-KOH pH 6.5

- d-Sorbitol (Fisher), Part # S459-500, $C_6H_{14}O_6$, MW=182.17 g/mol
- 500mM MES hydrate (ACROS Organics), Part # 172590250, MW=195.23 g/mol
- Potassium hydroxide pellets (85%, ACROS organics, part #206075000, MW 56.11)

500 mM d-Sorbitol and 500 mM MES were prepared in ultrapure water. Using a calibrated pH meter, KOH pellets were added to the MES solution until the solution reached pH 6.5. 1 part 500 mM MES-KOH pH 6.5 was added to 24 parts 500 mM d-Sorbitol. The resulting solution was transferred to an autoclave-safe container, the bottle caps were tightened one quarter turn and autoclaved 20 minutes on liquid setting. After autoclaving, the solution was allowed to cool before using.

PROTOCOL

2.5mL sterilized Reagent A was dispensed into disposable plastic cuvettes. Biomass was collected on filters of the desired pore size by gravity or vacuum filtration; nylon and GF/F filters were used for all experiments presented here. Immediately after filtration, the filter was placed into the cuvette using forceps, 4µl FDA was added (final concentration 10µM FDA), the sample was inverted to mix and incubated in the dark at 20ºC for 1 hour. At least 1 blank was included with each experiment. The blank should mimic the samples optically and chemically, but without viable organisms. Such a blank was prepared by treating sample water with 60ppm sodium hypochlorite and incubating for at least 30 minutes to kill all organisms before processing for Bulk FDA. If a more simple operation was desired, a "buffer only" blank was used in which FDA is added to incubation buffer alone. The bleach "killed" blank and "buffer only" blank generate similar fluorescence values. After a 1-hour incubation, the filter was removed using forceps. If GF/F filters were used, the filter was pressed against the side of the cuvette to squeeze fluid out of the filter, then the sample was centrifuged 30 seconds at 13,000 rpm to remove the pulp left behind by the GF/F filter. The fluorescence measurement was made (490 nm excitation, 513 nm emission) immediately after spinning down (or after removing the filter for nylon filters). A SPEX Fluoromax-2 spectrofluorometer and Turner Designs Aquafluor handheld spectrofluorometer were used for all measurements presented here.

Culture maintenance

Marine algal cultures used for the optimization experiments were reared at 25°C under natural light in 0.45 µm filtered seawater (FSW) with Guillard's marine enrichment solution added at F/2 concentration (50-fold dilution of stock solution). A complete listing of the cultured species is provided by Table 1. Experiments determining the relationship between biovolume, ATP and Bulk FDA using marine cultures were conducted using cells grown in FSW plus F/8 Guillard's (200-fold dilution of stock solution). F/8 concentration was used (rather than F/2) to prevent the precipitation of silicic acid. Freshwater cultures were grown under the same conditions in AlgaGro freshwater media (1x concentration, pH 7.8). Media was autoclaved 20 minutes in glass

culture flasks with cotton stoppers. Inocula were made into room temperature media by either pouring or pipeting. Algal cultures were collected from the MLML Biological Oceanography culture stock, Carolina Biological Supply, and the National Center for Marine Algae and Microbiotia (NCMA).

Table 1: Taxonomic listing of freshwater and marine algal cultures used in optimization experiments and to determine the relationship between fluorescein production, ATP and cell volume

Phylum	Class	Genus	Species
Heteroknotophyta	Coscinodiscophyceae	Thalassiosira	Pseudonana
Heteroknotophyta	Bacillariophyceae	Phaeodactylum	Tricornutum
Heteroknotophyta	Bacillariophyceae	Nitzschia	sp.
Haptophyta	Prymnesiophyceae	Pleurochrysis	Carterae
Chlorophyta	Chlorophyceae	Tetraselmis	sp.
Chlorophyta	Chlorophyceae	Dunaliella	sp.
Cyanobacteria	Synechococcophycideae	Synechococcus	sp.
Chlorophyta	Zygnemophyceae	Staurastrum	sp.
Chlorophyta	Zygnemophyceae	Cosmarium	sp.
Heteroknotophyta	Coscinodiscophyceae	Odontella	Mobiliensis
Heteroknotophyta	Coscinodiscophyceae	Coscinodiscus	sp.
Heteroknotophyta	Coscinodiscophyceae	Helicotheca	Tamensis
Heteroknotophyta	Coscinodiscophyceae	Helicotheca	Tamensis
Chlorophyta	Trebouxiophyceae	Chlorella	sp.
Chlorophyta	Chlorophyceae	Haematococcus	sp.
Heteroknotophyta	Coscinodiscophyceae	Thalassiosira	Weissflogii

Environmental Sample Collection

Natural water samples were collected around Moss Landing and southern Monterey Bay and used for optimization experiments, as well as for correlation with ATP and other live cell indicators. In most cases, seawater was sampled in polypropylene bottles or carboys at the surface in convenient locations, such as the MLML small boats dock. Offshore Monterey Bay samples were collected throughout the water column by deep-water CTD casts aboard the Research Vessel *Point Sur* March 28-29th, 2012, which were used for comparisons between fluorescein production and ATP concentration.

Flow cytometry

Flow cytometry was used often throughout the research presented here. A BD FACScan flow cytometer was used for experiments characterizing the intracellular pattern of fluorescein accumulation and leakage during incubation with FDA; these files were analyzed using Winmidi software. An Accuri C6 flow cytometer was used for all other experiments, usually to count live cells of natural samples and algal cultures; data analysis for these files was completed in the Accuri software package. High intracellular green fluorescence (detector FL1) after a 10 minute incubation with 10 µM FDA marks cells as alive. If a cell has a FL1 score higher than the set threshold value for a given size range of cells ($>10^5$ FL1 for cells 10-50 µm), it is scored as live. This threshold value was determined empirically using hypochlorite treated samples as negative controls, and untreated samples as positive controls. The number of live cells divided by the analysis volume yields live cell concentration. Measuring the live cell concentration enables one to load a known quantity of cells for a Bulk FDA experiment. Cultures of the small algal cells were counted by flow cytometry to determine cell concentration, while cultures of large cell size were counted by epifluorescence microscopy.

Epifluorescence microscopy

Numeric cell concentration of large-sized algal cultures could not be measured by flow cytometry, as large particles will clog the instrument. Therefore, in order to count large cells, 1-10 mL of the large-sized cultures were filtered onto 0.2 µm polycarbonate filters, placed on microscope slides and counted by epifluorescent microscopy. At least 3 slides were prepared for each culture and a minimum of 100 cells were counted per slide. A fraction of the filter area was counted; this count was extrapolated to the entire filtration area to calculate cell concentration.

Cell volume measurements: Coulter Counter

The cell volume of algal cultures was measured using a Z2 Beckman Coulter Counter. Marine cultures were diluted in FSW until an acceptable cell density was achieved (saturation indicator 0.3-.07). Freshwater cultures were diluted in AlgaGro freshwater media and 20% FSW was added to maintain a saline sample solution. 20%

FSW was shown to be the optimal salinity for both preserving the freshwater cell structure and maintaining the conductive solution that the Coulter Counter requires for signal transmission. The mean cell size, given as equivalent spherical diameter (ESD), was used rather than mode or median. A minimum of three samples were run for each culture, the average ESD of these three runs was used to calculate cell volume.

ATP

ATP was measured in tandem with fluorescein production (FP) from both natural samples collected in Monterey Bay and algal cultures. Biomass was collected by filtering sample water quantitatively onto GF/F or larger pore size nylon filters, just as in the Bulk FDA assay. Immediately after filtration, ATP was extracted by placing the filters into 13 mm diameter borosilicate test tubes containing 4 mL of boiling 20 mM Tris Buffer, pH 7.75. The filters were boiled at 100 ºC for 5 minutes, allowed to cool and then stored at -20°C until analysis.

ATP samples were analyzed using the Promega Enlighten™ ATP reagent kit. This kit uses a luciferin-luciferase reagent to measure ATP via the production of light according to the following reaction (Hamada et al. 1998):

$$ATP + D - Luciferin + O_2 \rightarrow Oxykuciferin + AMP + PPi + CO_2 + Light\ (560nm)$$

This light, termed luminescence, is proportional to ATP concentration. Luminescence was measured on a Turner Designs 20/20 ATP Luminometer. 100µL of the ATP extract was added to 50µL of luciferin-luciferase reagent in 3mL clear polystyrene uncapped tubes, mixed gently and measured on the ATP Luminometer using a 10-second integration time. ATP standards are included in the kit and were prepared using the same ratio of analyte volume to luciferin-luciferase reagent as used for samples. These standards were used to create a response factor to convert luminescence readings to ATP concentration (Hamada et al. 1998).

Pulse Amplitude Modulated (PAM) Fluorometry

Pulse Amplitude Modulated (PAM) fluorometry was used to measure variable fluorescence of algal cells, an indicator of physiological health. The commonly used ratio Fv/Fm expresses variable fluorescence (equation given below).

$$\frac{\mathrm{Fv}}{\mathrm{Fm}} = \frac{(F_{maximum} - F_{minimum})}{F_{maximum}}$$

Fv/Fm ranges from 0-0.8; low values (<0.2) indicate dead or unhealthy cells, intermediate values indicate cells stressed to some degree, while high values (>0.5) indicate robust, healthy cells capable of using incident light for photosynthesis. 3mL of cell suspension was transferred into round glass cuvettes and placed in the dark measuring chamber of the PAM fluorometer. Samples were dark adapted for at least 30 seconds and then excited with a high dose of light, termed a saturation pulse. Minimum fluorescence is measured immediately before this pulse, while maximum fluorescence is measured in response to this pulse.

RESULTS

Optimization Results

The preliminary results presented above show that extracellular FP is proportional to bulk living biomass. While this fundamental aspect of the assay is clear, there are several elements of the initial assay protocol which restrict its sensitivity, reproducibility and ease of use, most notably, the lack of a stable incubation buffer and non-biological fluorescein production. These issues, as well as other parameters involved in the protocol, made logical targets for optimization. The following elements on the Bulk FDA assay were investigated during the optimization process: 1) pH, 2) non-biological fluorescein production, 3) incubation buffer, 4) temperature, 5) incubation time, 6) incubation volume, 7) FDA concentration 8) sample mixing. The goal of the optimization process is to understand the effects of each aspect on the Bulk FDA technique, and to use this knowledge to improve the assay, making it as sensitive, precise, accurate, fast and simple as possible.

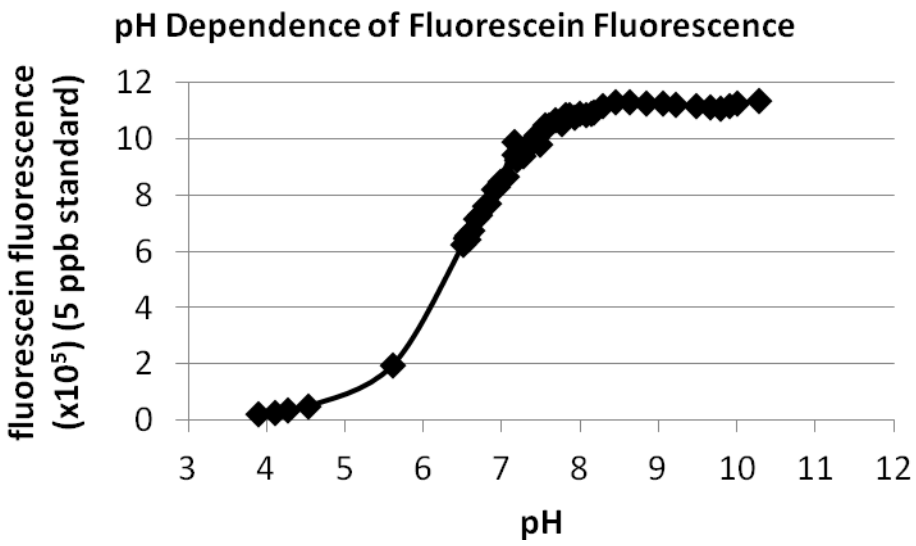
pH EFFECT ON FLUORESCEIN FLUORESCENCE

The intensity and wavelength of fluorescein fluorescence is very dependent on pH. Fluorescein fluorescence increases as pH increases, fluorescing most intensely at and above pH 8 (Figure 5). The increase in fluorescein fluorescence from pH 3.9 to pH 8.1 is roughly 50-fold. Fluorescein fluorescence remains constant between pH 8 and pH 10. Below pH 5, fluorescein fluoresces maximally when excited by 444 nm excitation light. Above pH 5, fluorescein fluoresces maximally when excited by 489 nm excitation light. Fluorescein fluorescence emission is at 515 nm across all pH levels.

ABIOTIC FLUORESCEIN PRODUCTION

One of the most important aspects of the optimization process was controlling and reducing the blank, the signal generated by the incubation conditions in the absence of live cells. This production of fluorescein is termed “abiotic” because it is produced by the chemical composition of the incubation buffer, not by the enzyme activity of live

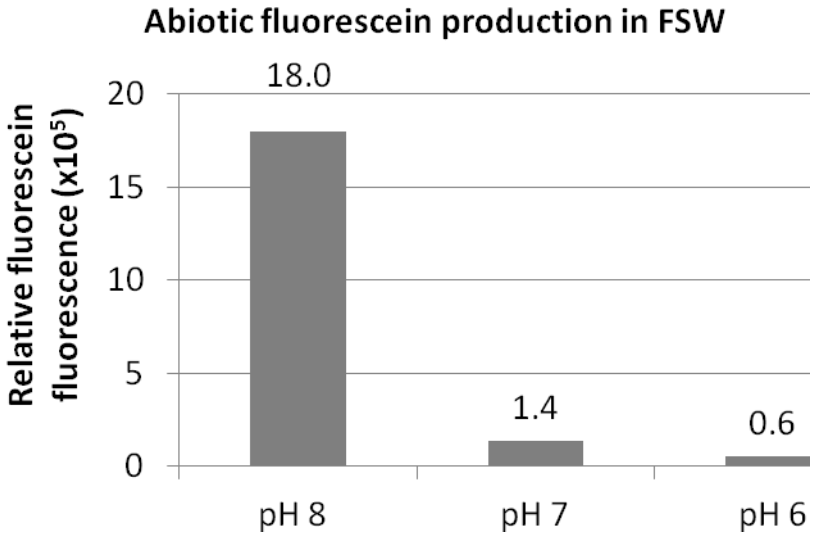
Figure 5: The pH dependence of fluorescein fluorescence



cells. When the Bulk FDA incubation is carried out using filtered, autoclaved seawater (FSW) as the incubation buffer, very high FP is observed in the cell-free blank. Lowering the pH of FSW with hydrochloric acid results in a large reduction of blank fluorescence. Lowering the pH from 7.8 to 6.0 causes a 32-fold reduction in abiotic FP (Figure 6). The fluorescence live cell samples decreases at lower pH, yet does so less than the blank, resulting in much improved signal/noise ratios in acidified FSW relative to unadjusted FSW. Some of the reduction in the blank when using acidified FSW is due to the decrease in intensity of fluorescein fluorescence due to lowered pH alone. However, the reduction in blank fluorescence when using FSW at pH 7.8 to acidified FSW at pH 6.0 (32-fold) exceeds the reduction of fluorescence from sodium fluorescein standards observed at pH 7.8 and pH 6.0 (~2.5-fold) (Figure 5). This suggests that the decreased efficiency of fluorescein fluorescence at low pH is only a partial contributor towards the decrease in abiotic FP in FSW pH 6.0. The chemical composition of FSW at pH 7.8 hydrolyzes more FDA than at pH 6, resulting in high abiotic FP values in the absence of esterase enzymes.

Acidified, filtered and autoclaved seawater is an acceptable Bulk FDA incubation solution for providing relative FP measurements from marine organisms. However, the pH of FSW acidified with hydrochloric acid is not stable when exposed to air; the pH will

Figure 6: Abiotic FP resulting from bulk FDA incubations using non-acidified (pH 8) and acidified (pH 7 and 6) FSW as the incubation buffer.



rise continuously until it reaches normal seawater pH (pH 7.8), therefore, the actual pH during an incubation with acidified FSW cannot be predicted. The mass of fluorescein produced by a sample is determined by relying on fluorescein standards of known pH. If the pH is not known at the time the fluorescent measurement is made, the fluorescent result cannot be converted to fluorescein concentration; the actual mass of fluorescein produced by the cells being assayed cannot be determined. Acidified seawater thus indicates relative FP and does not provide a standardized incubation condition, which greatly compromises reproducibility of the assay from day to day. An incubation buffer that maintains constant pH is clearly needed.

INCUBATION BUFFER OPTIMIZATION

The ideal buffer for the Bulk FDA assay will possess the following qualities:

1) Low abiotic fluorescein production (low blank)
2) High and consistent fluorescein production per unit living biomass
3) Buffered to maintain constant pH
4) Autoclavable to maintain sterility during storage
5) Compatible with freshwater and marine organisms

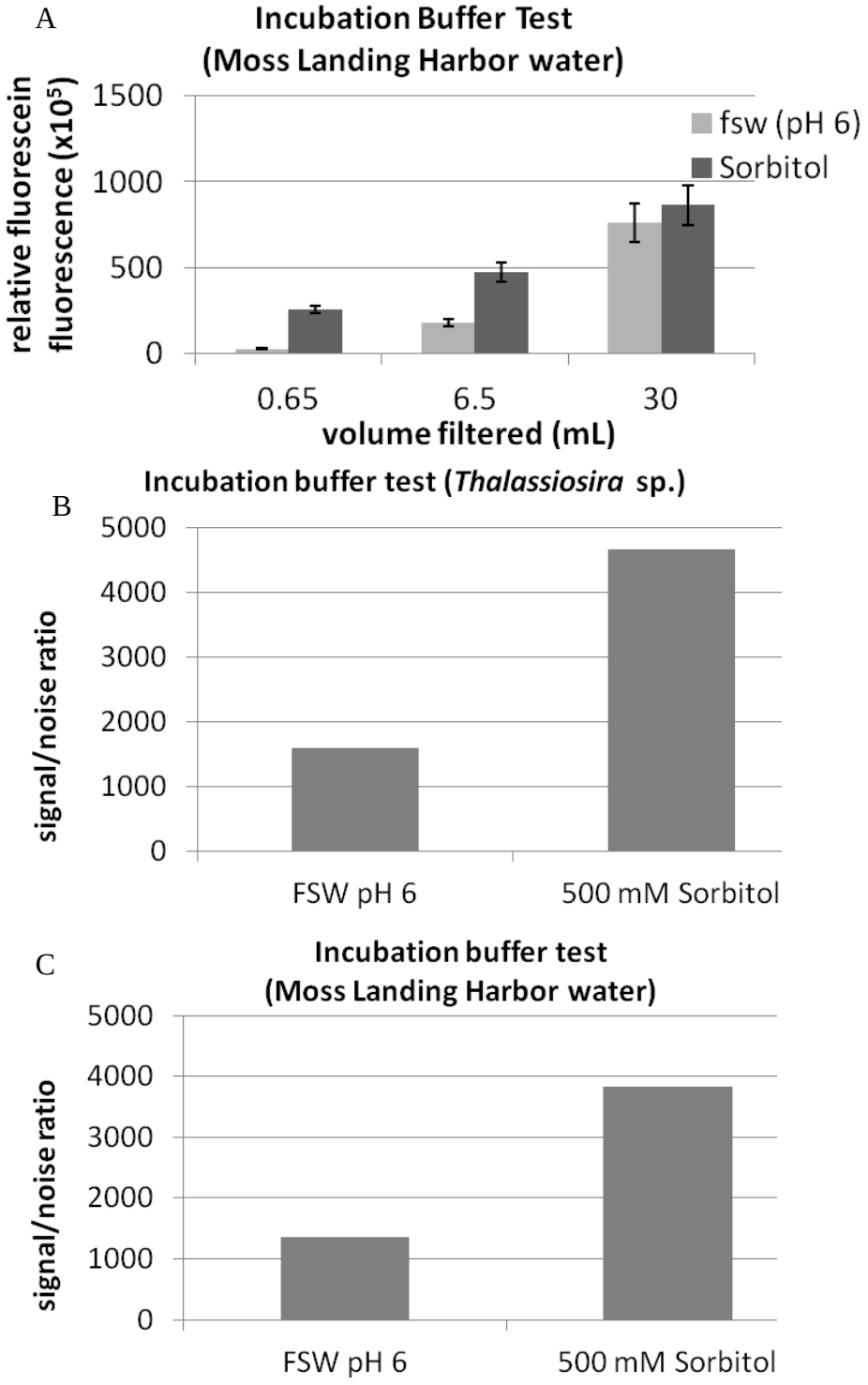
Many potential incubation solutions, often containing supplementary reagents, were tested during the optimization process; many of the experiments involving unsuccessful incubation buffers are not presented here. While the selection of test solutions was guided by all the criteria listed above; final selection was based on the signal to noise ratio, given by criteria 1 and 2, so as to provide the best sensitivity for detection of low viable biomass ranges. Commonly used buffered solutions, such as phosphate, citric acid and tris based buffers, were tested at many concentrations and pH levels. Artificial seawater solutions were tested. Supplemental reagents, including DTT and EDTA, were tested as additives to the buffers in attempts to help facilitate enzymatic activity. Reagents designed to permeabilize cell membranes and enhance leakage were tested, such as detergents (Triton X-100) and lysozyme extracts. None of the supplementary reagents tested improved the assay; most led to a drastic reduction in FP. Only one of these solutions, d-sorbitol, represented an improvement over acidified FSW at satisfying the optimization criteria.

d-Sorbitol is identified by Welschmeyer and Maurer (in prep, 2013) as a promising Bulk FDA incubation solution. d-Sorbitol, also called Glucitol, a sugar alcohol often used as a sugar substitute in food items, can serve as an osmoproctectant in the culturing of algae. Experiments confirm that 500mM sorbitol gives a larger signal and lower abiotic FP than acidified FSW when Bulk FDA incubations are carried out with fixed quantities of both freshwater and marine algal cultures, as well as natural samples (Figure 7a), resulting in higher signal/noise ratios in 500mM sorbitol relative to acidified FSW (Figure 7b and 7c). The signal/noise ratio improves most drastically, as much as 6-fold, when a small quantity of cells are loaded, suggesting that sorbitol is a more effective incubation solution than acidified FSW for detecting low biomass levels. Sorbitol is not, however, buffered to maintain constant pH.

BUFFERING THE INCUBATION SOLUTION

The pH of 500mM sorbitol, when it is not adjusted or buffered, varies between 3.7 and 5.4. Freshly prepared sorbitol is pH 5.4; the pH will typically decrease over time. The pH of sorbitol will change drastically if more basic substances are added, such as seawater (Figure 9). Because a small, and likely variable, volume of seawater is retained

Figure 7: Experiments comparing the use of acidified FSW vs. 500mM sorbitol as bulk FDA incubation buffers



on filters used to collect cells, the incubation solution must be buffered against pH changes caused by seawater retained on the filter. For example, GF/F filters retain approximately 100µL of fluid after vacuum filtration. The optimal buffering agent must efficiently stabilize the pH of sorbitol, but it must also create a solution that is optimal in regard to maximizing signal/noise ratio and the other criteria listed above.

The buffers MES, MOPS and HEPES were tested in sorbitol at several concentrations and pH levels. The signal to noise ratio in each buffered solution was used as the primary criteria for selecting the optimal buffering agent. 10,000 *Tetraselmis* sp. cells were filtered onto GF/F filters and incubated in each solution with 10µM FDA. The blank consisted of a GF/F prepared the same way as above, but with autoclaved FSW substituted for the algal culture. FP from the *Tetraselmis* sp. samples divided by FP from the blank yields the signal/noise ratio. 500mM sorbitol stabilized with MES-KOH pH 6 gave a lower blank and a higher signal to noise ratio than solutions buffered with HEPES or MOPS (Figure 8).

The buffering capacity of the buffers MES, HEPES and un-buffered Sorbitol was determined by titrating filtered seawater (pH 7.9) into the solution and monitoring the resulting pH. Filtered seawater was used to mimic the pH perturbation expected during an incubation. 500mM sorbitol with 2 mM MES-KOH pH 6 changed less in response to the addition of filtered seawater than 2mM HEPES pH 6 and unbuffered sorbitol (Figure 9).

500mM sorbitol with MES-KOH pH 6 showed the greatest buffering capacity while yielding the lowest blank and highest signal to noise ratio of the buffers tested above. The next step in the optimization process was determining the ideal concentration of MES-KOH. Experiments were set-up to answer which MES-KOH pH 6 concentration would give the highest signal to noise ratio, lowest blank, and would buffer most effectively. Experiments comparing 2, 10, and 50 mM MES-KOH pH 6 in 500mM sorbitol showed roughly equivalent signal to noise ratios. Titrating all three buffers with bleach (which raises pH) showed that the buffering capacity of the solution varied as a function of the MES concentration; low concentrations of MES produced a weakly buffered solution, while raising the concentration of MES to 20 mM resulted in a solution

Figure 8: The signal/noise ratios resulting from bulk FDA incubations in 500mM sorbitol buffered with 2mM, pH 6 MES, MOPS and HEPES

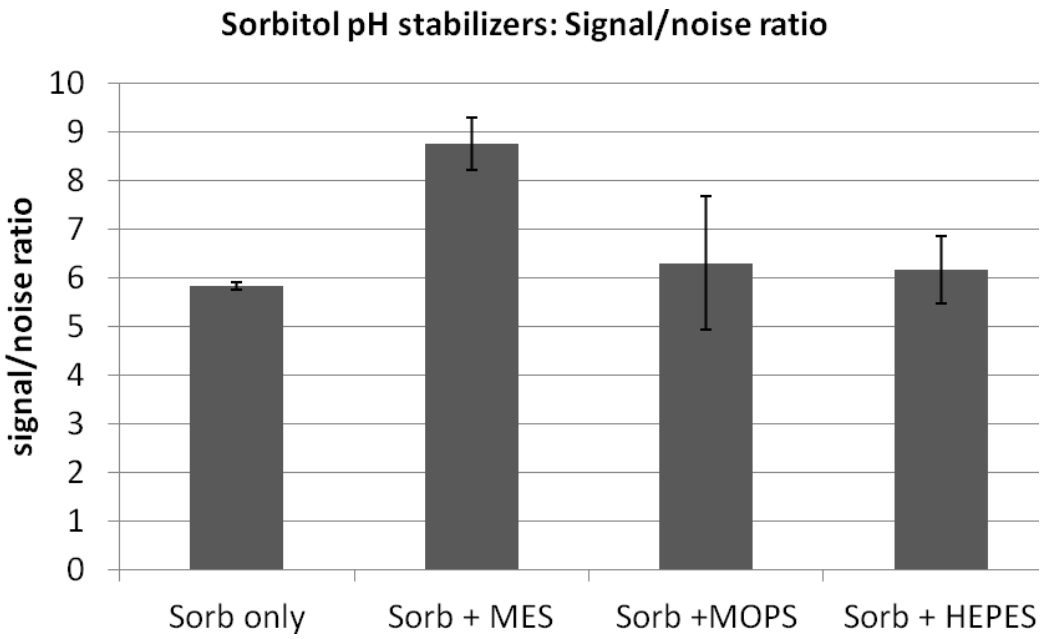
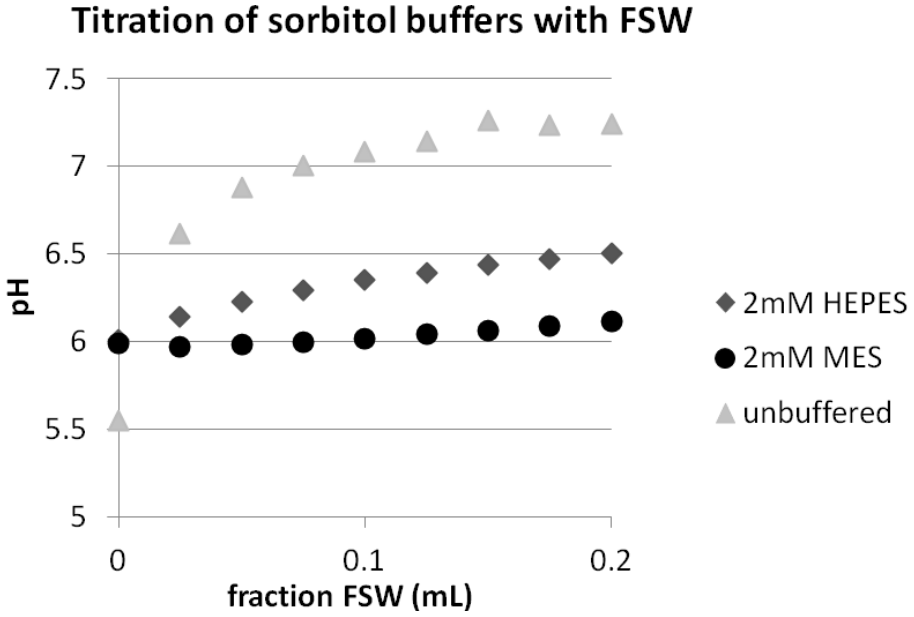


Figure 9: The buffering capacity of 500mM sorbitol in response to FSW without a buffering agent, buffered with 2mM MES and with 2mM HEPES

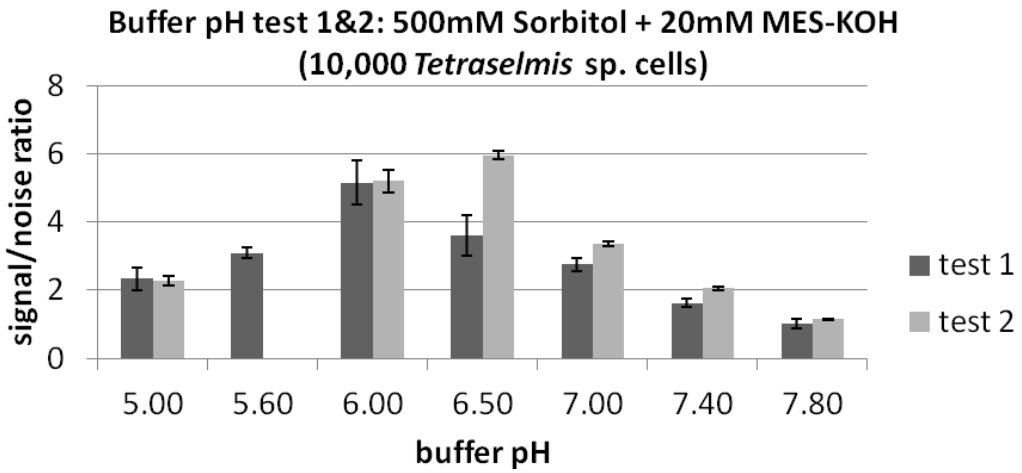


resistant to pH change. Based on these results, the optimal MES concentration was determined to be 20 mM.

In the experiments presented above, 500mM sorbitol + 20mM MES pH 6.0 gave higher signal/noise ratio and buffering capacity than lower pH levels. Subsequent experiments were performed to determine the optimal buffer pH by preparing 500mM sorbitol with 20mM MES adjusted with KOH to pH 5-7.8. In one instance, pH 6.0 gave a 1.4-fold higher signal/noise ratio than pH 6.5 using cultured *Tetraselmis* sp. cells (9µm ESD) (Figure 10a) collected on GF/F filters. However, repeating this experiment under the same conditions showed that pH 6.5 buffer gave a 1.15-fold higher signal/noise ratio than pH 6.0 (Figure 10a). An experiment using a fixed quantity of the larger sized (33µm ESD) cultured alga *Odontella* sp. collected on 10µm nylon filters gave a 1.19-fold higher signal/noise ratio in pH 6.5 buffer relative to pH 6.0 (Figure 10b). Tests using San Francisco Bay ballast water collected on 10µm nylon filters have shown that pH 6.5 buffer gives 1.7-fold higher signal/noise ratios than pH 6.0 buffer, on average (individual values: 1.65, 1.88, 1.62, 1.66) (Figure 10c). The difference between the signal to noise ratios using buffer at pH 6.0 and 6.5 is not large, as the fluorescence of both the blank and the signal increase with pH in close to a proportional manner. The raw fluorescence of fluorescein increases by 1.8-fold by increasing the buffer pH from 6.0 to 6.5, as determined using sodium fluorescein standards (Figure 6). Because the same fluorescein concentration is more easily detected by spectrofluorometry at pH 6.5, less sensitive instrumentation is needed. For these reasons, pH 6.5 has been selected as the optimal buffer pH.

Figure 10: Incubation buffer pH tests

A



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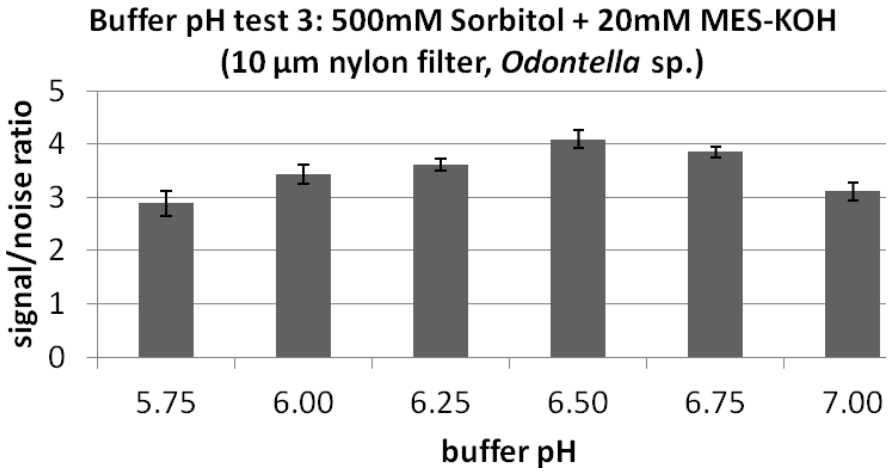
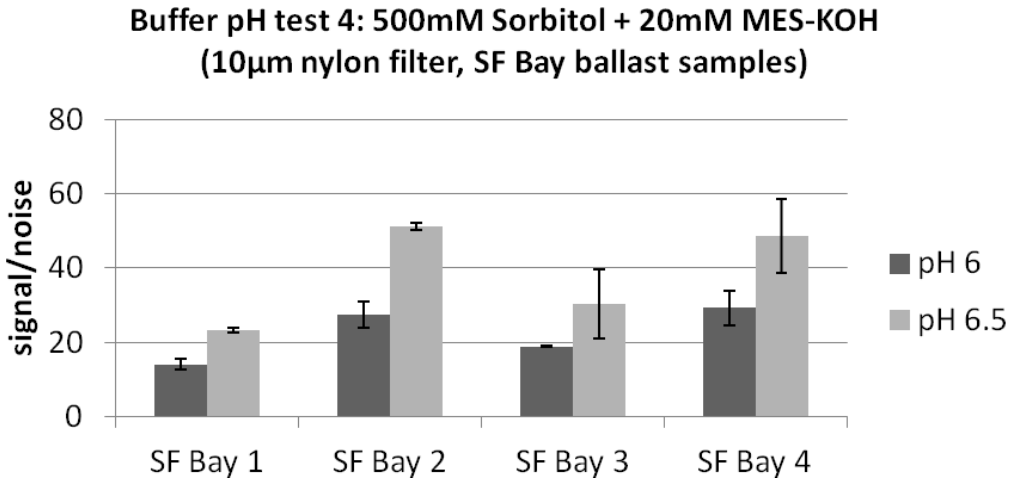


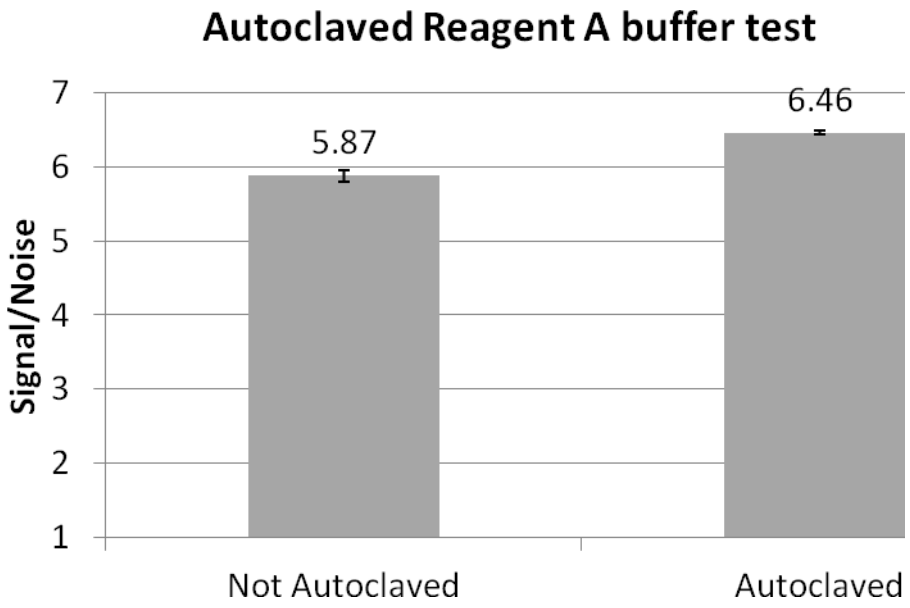
C



STERILIZING THE INCUBATION SOLUTION

The optimal buffer must be able to be autoclaved to maintain sterility without changing the pH or effectiveness of the buffer. Experiments comparing freshly made 500mM sorbitol + MES-KOH pH 6 with freshly made and then autoclaved solution show that the autoclaved incubation solution has a slightly lower blank, probably because bacterial contamination has been eliminated, resulting in slightly higher signal/noise ratios in autoclaved buffer (Fig 11). The pH of the solution does not change with autoclaving.

Figure 11: Signal/noise ratios in autoclaved vs. non-autoclaved bulk FDA incubation buffer.



TESTING THE OPTIMIZED INCUBATION BUFFER

The final stage of incubation solution optimization includes ensuring that the solution can accommodate both freshwater and marine organisms. Pulse Amplitude Modulated fluorometry (PAM) was utilized to gain insight into the health of marine and freshwater algal cells during a Bulk FDA incubation. The following incubation buffers were tested: 500mM sorbitol+ 20mM MES-KOH pH 6.5, a commercially supplied

potassium phosphate buffer with MgCl, and the native media for freshwater (AlgaGro) and marine (Guillard's marine enrichment solution, F/2 concentration) cultures. *Tetraselmis* sp. and *Coscinodiscus* sp., both marine cultures, and *Haematococcus* sp., a freshwater alga, were gravity filtered and gently re-suspended in the various buffers. Variable fluorescence was measured immediately before and after re-suspension in buffer, after 1 hour, and at an extended time point which varied between 2 hours and 2 hours 35 minutes (Figure 12, Table 2). All three cultures showed very little immediate change in Fv/Fm in the incubation buffers and native media. *Coscinodiscus* sp. showed some decline in Fv/Fm in the sorbitol incubation buffer at the 1-hour and >2-hour time point relative the initial reading, while showing very little change in Fv/Fm over time after re-suspension in the marine media and a significant decrease in phosphate buffer. *Tetraselmis* sp. did not show any reduction in sorbitol buffer nor in marine media, while it did show a significant reduction over time in phosphate buffer. Conversely, the reduction in Fv/Fm of the freshwater alga *Haematococcus* was more pronounced in sorbitol buffer than in phosphate buffer, while no significant change was observed in freshwater media.

The same re-suspended samples that were used to make the PAM measurements described above were also inspected microscopically to see if the cells showed any morphological change after being re-suspended in the re-suspension solutions (Figure 13). *Tetraselmis*, a mobile, flagellated organism, stopped swimming in the phosphate buffer, yet remained swimming in both the sorbitol buffer and in marine media. This result is consistent with the PAM data, as the Fv/Fm of *Tetraselmis* was markedly reduced in the phosphate buffer. No visual effect was observed in the re-suspended solutions of *Coscinodiscus* and *Haematococcus*, as both species remained intact without any obvious morphological change in all three solutions.

Results of the re-suspension experiment suggest that 500mM sorbitol + 20mM MES-KOH pH 6.5 causes more osmotic stress to freshwater organisms than to marine organisms. However, the freshwater alga, while showed reduced variable fluorescence, is still intact, viable and capable of producing fluorescein at close to the expected rate.

Figure 12: The variable fluorescence, measured by PAM fluorometry, of freshwater and marine algal cultures re-suspended in Reagent A and in native media

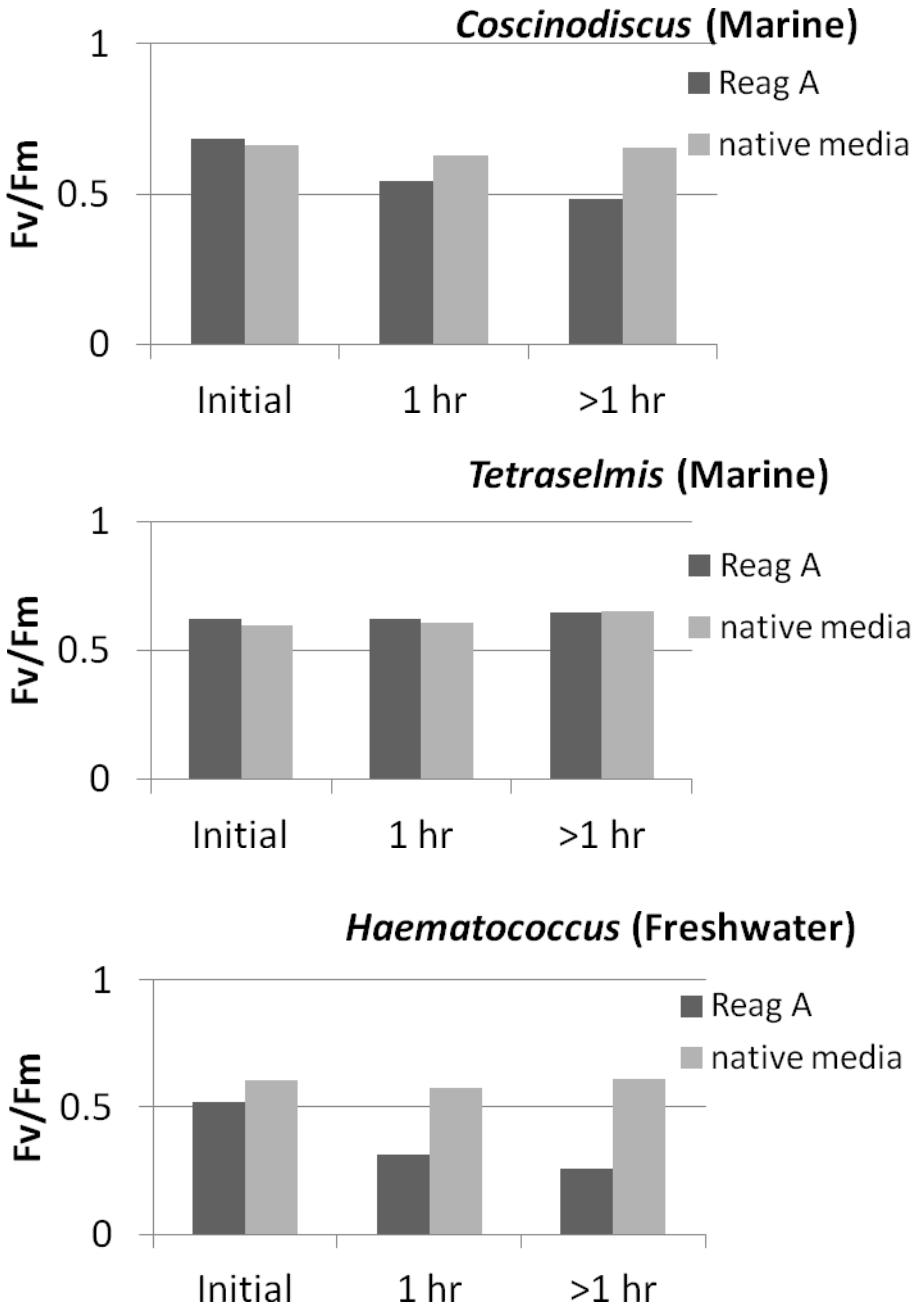
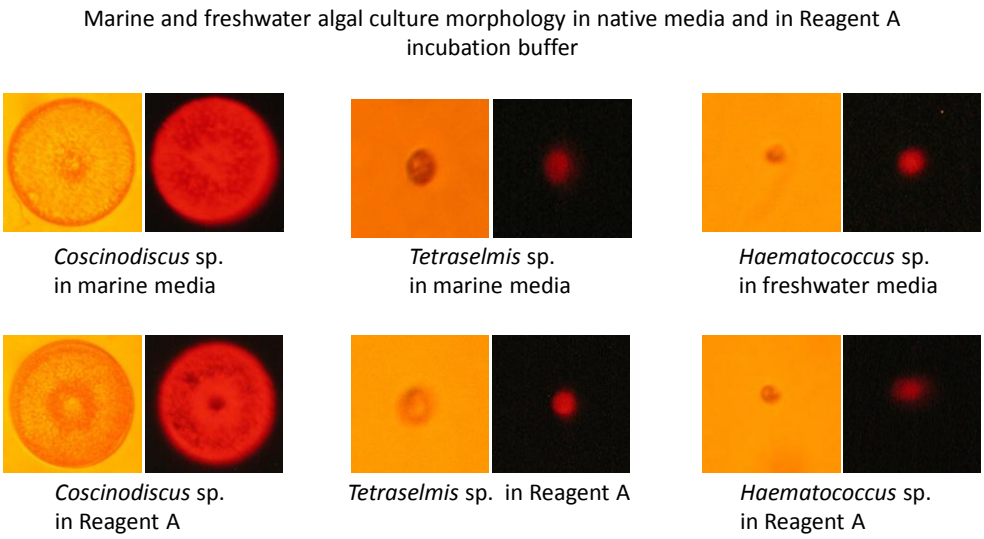


Table 2: Fv/Fm values from re-suspension of marine and freshwater algal cultures in native media, Reagent A, and Phosphate buffer at initial, 1 hour and 2-2.5 hour time points

Genus	Re-suspension fluid	Initial Fv/Fm	1 hour Fv/Fm	> 2 hour Fv/Fm
Coscinodiscus **(marine)**	Native media	0.66	0.63	0.65
	Reagent A	0.69	0.54	0.48
	Phosphate buffer	0.60	0.37	0.21
Tetraselmis **(marine)**	Native media	0.60	0.62	0.65
	Reagent A	0.62	0.62	0.65
	Phosphate buffer	0.45	0.24	0.14
Haematococcus **(freshwater)**	Native media	0.61	0.57	0.61
	Reagent A	0.52	0.32	0.26
	Phosphate buffer	0.67	0.55	0.57

Figure 13: Bright field and epifluorescent microscope photographs of algal cultures re-suspended in their native media and Reagent A.



Therefore, 500mM sorbitol stabilized with 20mM MES-KOH pH 6.5, hereafter called Reagent A, has been selected as the recommended reagent for Bulk FDA experiments.

TEMPERATURE EFFECT

The assay relies on the activity of esterases, lipases and proteases which, like all enzymes, are sensitive to temperature changes. Generally, enzyme activity increases with temperature up to some temperature optimum; elevating the temperature past this optimum will begin to irreversibly denature enzymes, resulting in lower enzyme activity. The optimal temperature and temperature range that can be tolerated vary considerably among enzymes, but are generally close to the ambient temperature of the organism or environment in which the enzymes are found. In biological systems involving enzymes, a predictable response to temperature is often observed which can be described by the Q10 coefficient (James 1953), which describes the rate of change in a biological or chemical system as a result of increasing the temperature by 10°C. The Q10 coefficient is calculated according to the following equation, where R_1 and R_2 are the rates of activity at each respective temperature (T_1 and T_2):

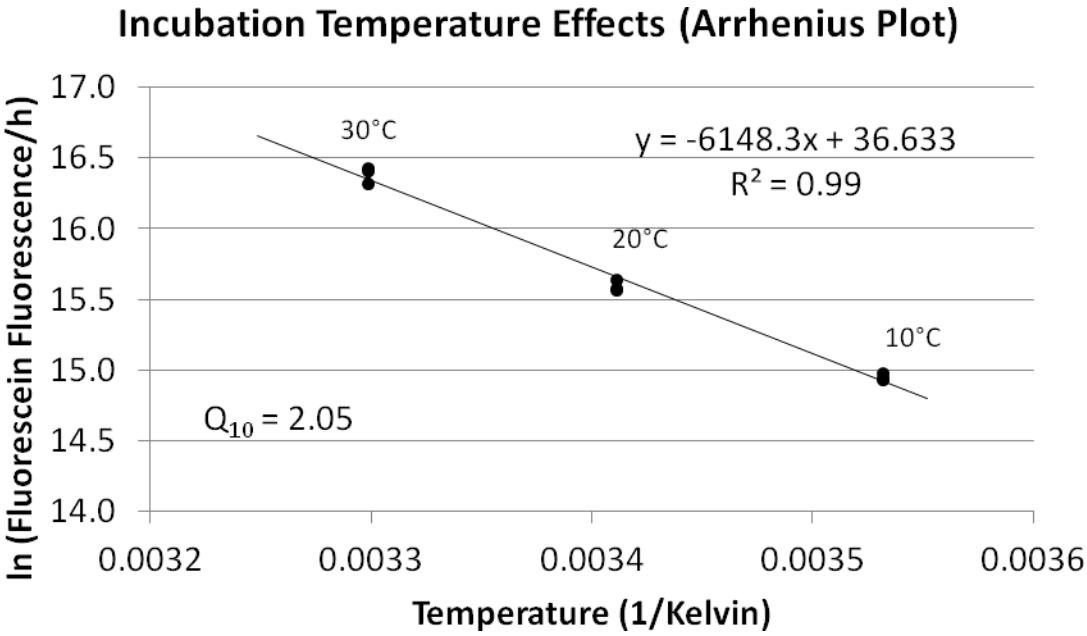
$$Q_{10} = \left(\frac{R_2}{R_1}\right)^{\frac{10}{T_2 - T_1}}$$

For example, if increasing the temperature from 10° C to 20° C results in a 2-fold increase in enzyme activity, $Q_{10} = 2$.

Elevated temperatures increase the EA of cells or organisms being assayed and therefore, increase FP. Controlling the ambient temperature is an important aspect of maintaining standardized incubation conditions. Experiments presented here, including those which enable the prediction of live cell concentration from FP, have been carried out at 20°C. The Bulk FDA technique has been designed as a portable viability assay that must function predictably across the range of temperatures likely to be encountered in the field. If the assay is preformed at very high or low temperatures, a correction factor must be applied to compensate for the change in EA due to temperature. This correction factor was determined empirically. Bulk FDA incubations in acidified FSW

(pH 6) were loaded with a fixed quantity of cultured *Tetraselmis* sp. cells and incubated at 10, 20 and 30°C. The resulting data was plotted on an Arrhenius plot (Figure 14) which shows the significant and predictable relationship between temperature and FP/cell. The Q_{10} coefficient of 2.05 indicates a 10° C increase will cause a 2.05x increase in FP, which can be used as a correction factor to adjust fluorescent results generated at high or low temperatures. For example, if the assay is preformed at 30°C, divide the final FP value by 2.05 to compare with incubations preformed at 20°C.

Figure 14: An Arrhenius plot shows the predictable relationship between FP by cultured *Tetraselmis* sp. cells and temperature



INCUBATION TIME

The Bulk FDA technique measures the rate of extracellular FP and is typically displayed in the following units:

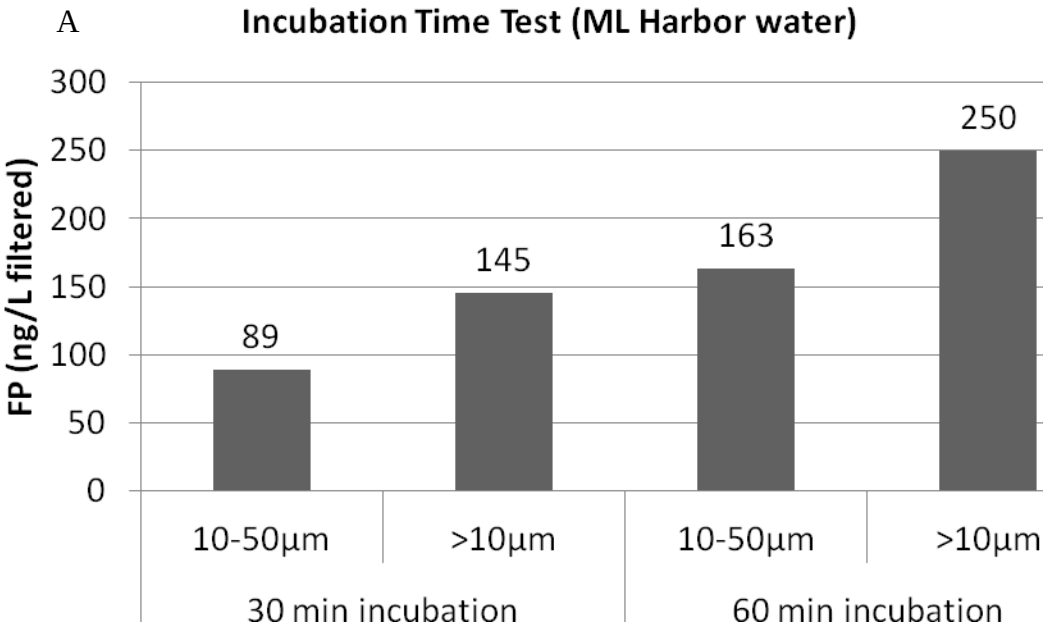
$$\frac{\text{fluorescein produced (ng)}}{\text{hr. volume filtered (L)}}$$

In a typical Bulk FDA incubation, this rate is observed simply as the fluorescein signal at the end of a set incubation time (1 hour). The rate of FP can be studied more

carefully by making multiple extracellular fluorescein fluorescence measurements over an incubation period following the addition of FDA, as in the characterization of intracellular and extracellular FP experiment previously presented. Similar experiments using both cultured algal cells and natural samples have shown that extracellular FP proceeds at close to a constant rate; i.e. doubling the incubation time will result in close to double the final fluorescein concentration. Figure 15a shows that 13% more fluorescein is produced in the first 30 minutes than in the second 30 minutes of a 1-hour incubation with 200mL Moss Landing harbor water. The signal to noise ratio increases as incubation time increases (Figure 15b), because the fluorescence from a sample increases at a greater rate than the fluorescence of a blank. In light of these results, 1-hour incubations are recommended whenever possible; slight deviations from a 1-hour incubation can be corrected for assuming a linear production of extracellular FP over time.

Figure 15: Effects of incubation time on a) FP and b) signal/noise ratios of size fractionated Moss Landing harbor water and *Tetraselmis* sp. cells, respectively.

A



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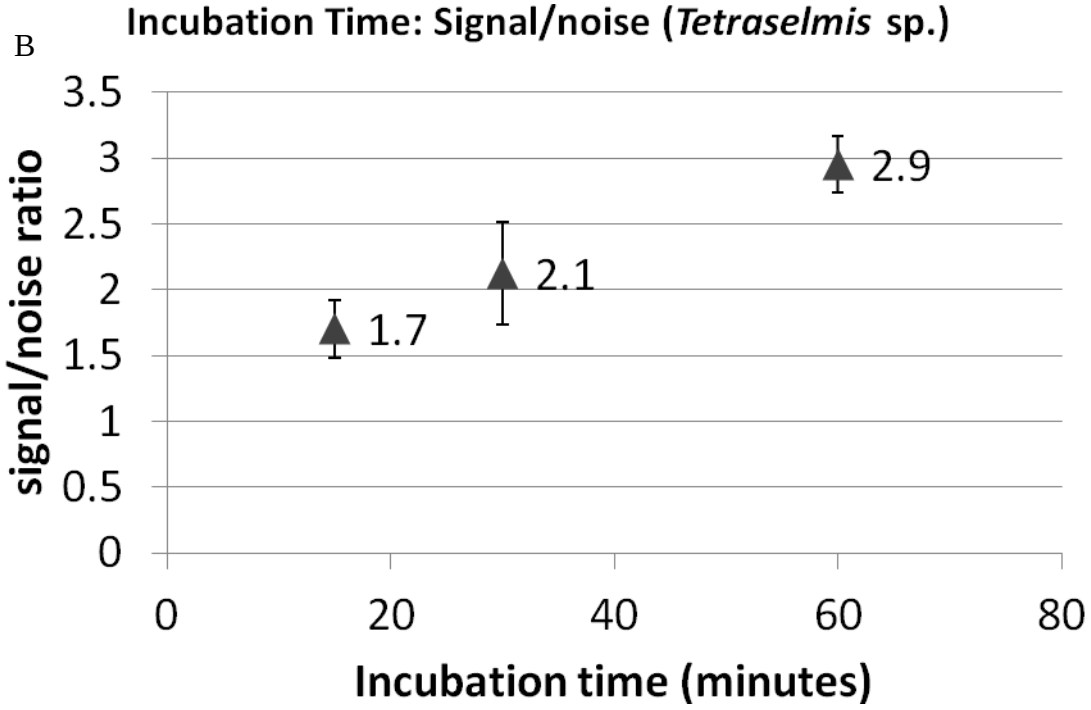
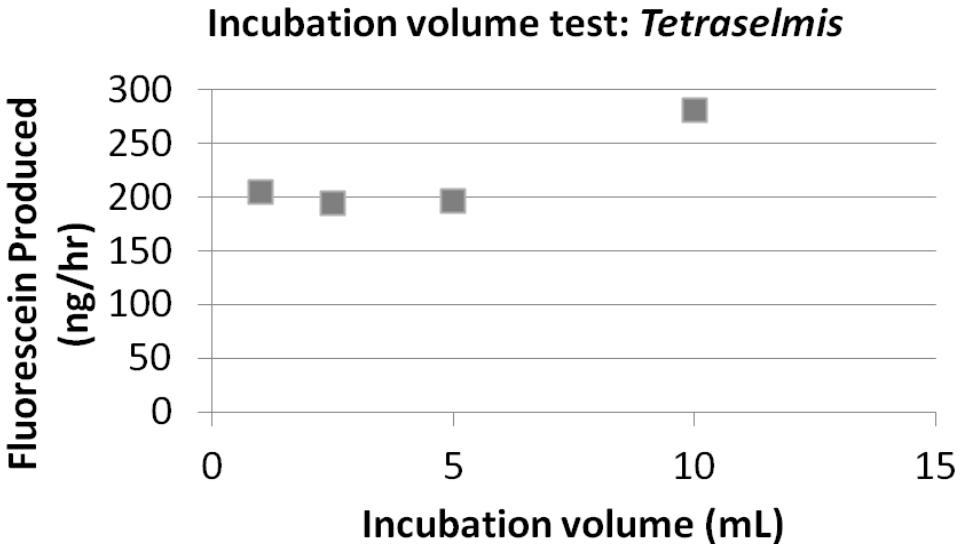


INCUBATION VOLUME

While the mass of fluorescein produced is the key aspect of the Bulk FDA technique, the fluorescence at the end of an incubation in Reagent A is an indicator of fluorescein concentration (µg/L). The user must multiply by the incubation volume to convert fluorescein concentration to the mass of fluorescein produced. Experiments were devised to investigate if changes to the incubation volume would result in changes to FP rates by a fixed quantity of cells, or if the same mass of FP would be produced regardless of incubation volume. Bulk FDA incubations were performed using a constant quantity of cultured *Tetraselmis* sp. cells and 10µM FDA. An incubation volume of 10mL yielded 42% higher mass FP (µg/hr) than the mean of the lower incubation volumes tested (Figure 16). While normalized FP was highest with 10mL incubation volume, fluorescein concentration was highest in the low incubation volume samples. Relatively constant mass FP rates were observed among samples with 1, 2.5 and 5mL incubation volumes. Increasing incubation volume provides a greater pool of FDA available for hydrolysis, which may explain the increase in FP using 10 mL incubation volume. These results show that this effect is not significant, especially when incubation volumes between 1 and 5mL are used. The Bulk FDA assay is therefore adaptable to varying incubation volumes to without losing comparability among FP results.

Figure 16: The effect of incubation volume on FP by cultured *Tetraselmis* sp. cells.



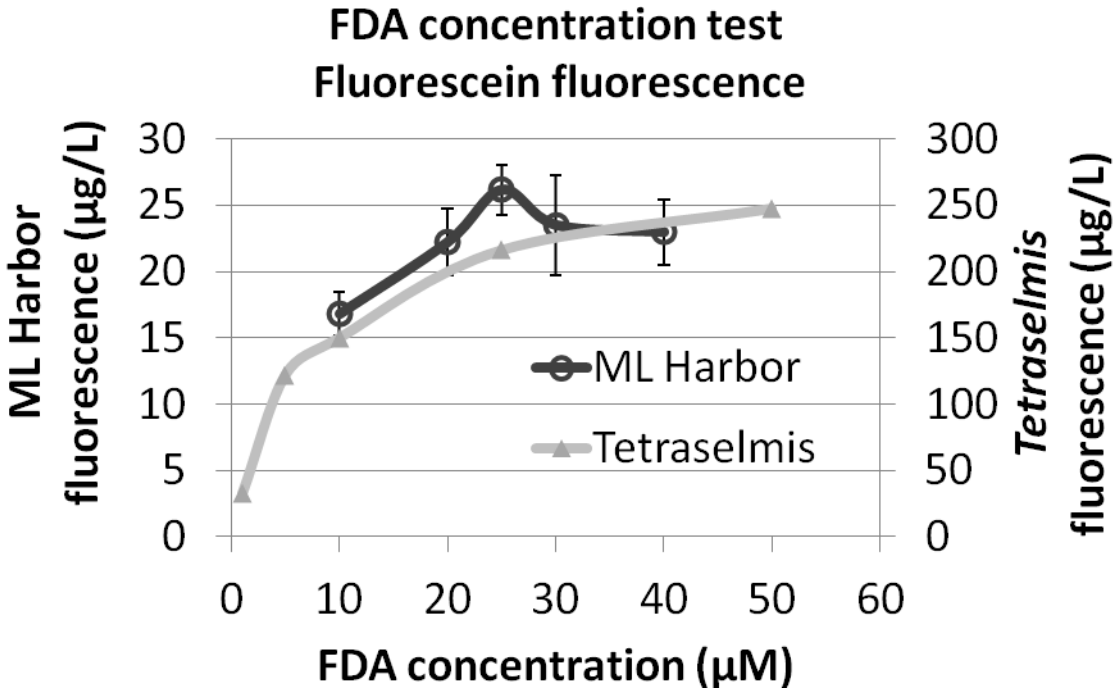
FDA CONCENTRATION

When enzyme activity is measured, it is customary to keep the substrate of the enzymes being assayed in excess. Using this approach, the amount of substrate that is converted to product is proportional to enzymatic activity only, rather than being proportional to the substrate concentration and enzyme activity. One can determine the concentration at which the substrate becomes limiting for a given enzymatic assay by titrating the substrate concentration, incubating in the presence of enzyme and recording the concentration of product formed at each substrate concentration. A plot of substrate concentration vs. product formation will show the concentration at which substrate is limiting. At areas of the plot where increasing the substrate concentration causes an increase in product formation, the substrate is limiting, as the amount of product formation depends on both the enzyme activity and the substrate concentration. When substrate is in excess, further increases in substrate concentration do not result in an increase in product formation; the amount of product formation depends only upon enzyme activity.

To test the effect of FDA concentration, Bulk FDA experiments were performed using cultured *Tetraselmis sp*. cells and Moss Landing harbor. Constant biomass levels were loaded onto GF/F filters and incubated in Reagent A buffer across a range of FDA concentrations (1-500 µM FDA) (Figure 17). To control for any effect of the solvent in which the FDA stock is dissolved (DMSO), varying concentrations of stock FDA were prepared such that the same volume of DMSO (10µl) was added to each sample. Increasing the FDA concentration results in an increase in product formation until an FDA concentration of about 25µM, after which increasing the concentration above 25µM does not cause an increase in FP. This suggests that FDA is in excess at around 25µM; FDA would therefore ideally be added at a concentration greater than or equal to 25µM. However, the signal/noise ratio is highest at 10µM (Figure 17b). Breeuwer et al., 1995 report that the maximum concentration of FDA that can be dissolved in aqueous solvents is 10µM; if more FDA is added a visible precipitate will form. Precipitated FDA will not serve as a substrate for esterase activity unless it re-dissolves. Because the re-dissolution of FDA is a potentially variable process that could decrease the precision of the assay,

Figure 17: The effects of FDA concentration on FP by *Tetraselmis* sp. cells and Moss Landing harbor water.

A



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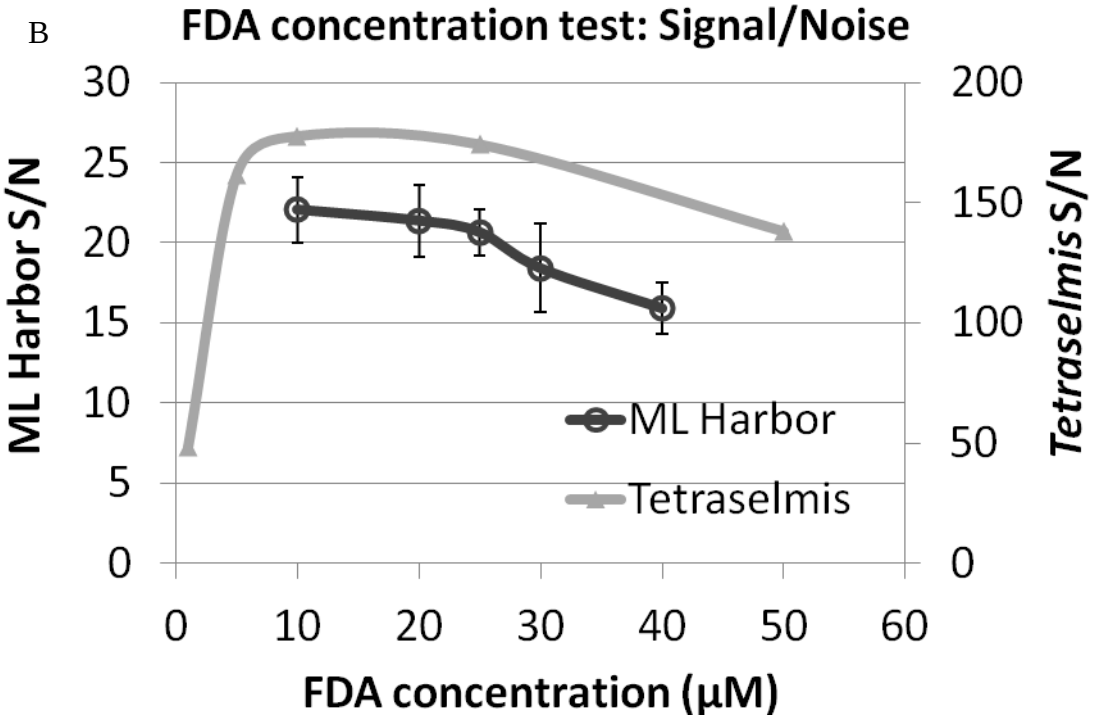


and greatest sensitivity (signal/noise) is achieved at 10µM FDA, 10µM has been selected as the optimal FDA concentration.

When the Bulk FDA assay is carried out using 10µM FDA and an incubation volume of 2.5mL, 25nmols of FDA are added. A typical, 1-hour Bulk FDA incubation using 10µM FDA, will produce between 0.038 - 7.5 nmols. The lower limit of this range (0.038nmols FP) corresponds to a signal 5-fold higher than the blank. The upper limit (7.5nmols FP) corresponds to the highest fluorescence that can be detected reliably using the SPEX Fluoromax 2 fluorometer; loading biomass at high levels which results in fluorescence above this limit is avoided. This shows that between 0.15-30% of the FDA added is converted to fluorescein, based on a 1:1 stoichiometric relationship between FDA and fluorescein.

SAMPLE AGITATION BEFORE AND DURING THE BULK FDA INCUBATION

The effects of vortexing samples at the beginning and end of the Bulk FDA incubation were investigated, as well as the effects of continually agitating the samples during incubation. Vortexing serves to quickly and efficiently homogenize the FDA within the incubation buffer, as well as removing cells off the filter that was used to harvest cells. Figure 18 shows that vortexing for 5 seconds actually lowers the FP produced by natural samples (Moss Landing harbor water) by roughly 25% relative to using multiple gentle inversions to mix each sample. Vortexing may harm cells in some way and, therefore, is not recommended.

A test of constant mixing during the incubation vs. mixing only at the start of the incubation was performed using cultured *Tetraselmis* sp. cells. Samples were mixed by inverting only and left to incubate without agitation, while identical samples were mixed by inverting and then placed on a mechanical mixer, which agitated samples using a back and forth rocking motion. 5 different biomass levels were loaded and 4 of the 5 showed greater FP without rocking; FP was an average of 73% higher in non-agitated samples (Figure 19). Agitation during the Bulk FDA incubation is not recommended.

Figure 18: The effects of vortexing after adding FDA and before making the fluorescent measurement

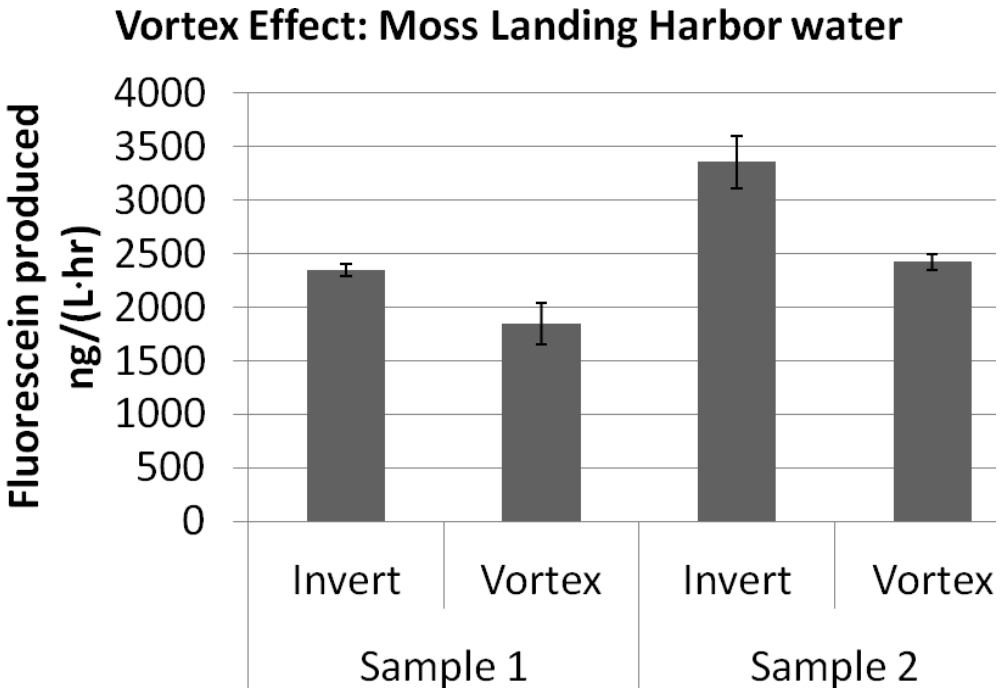
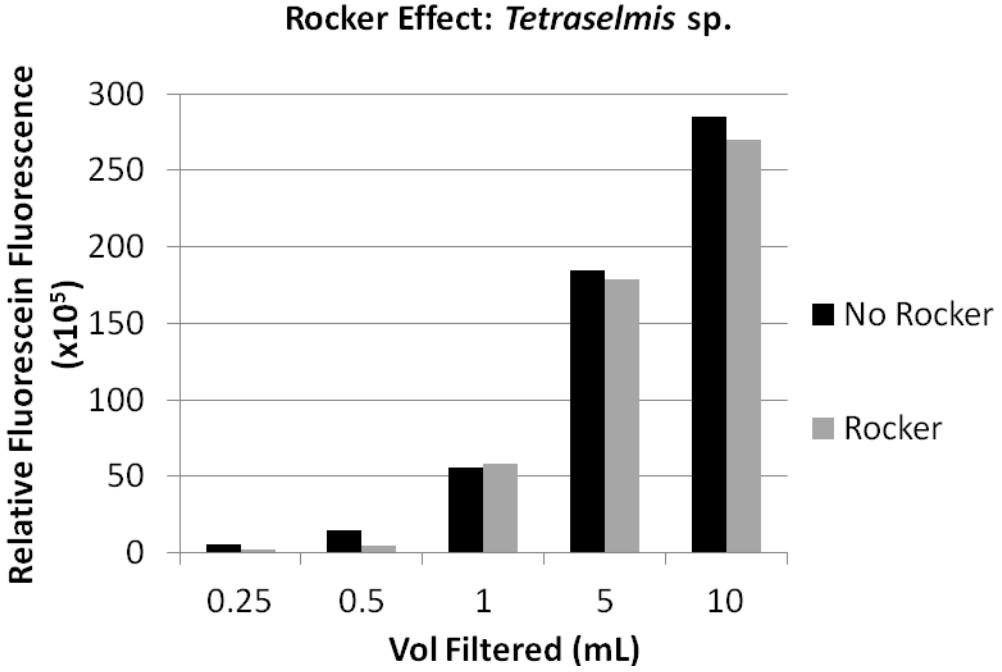


Figure 19: The effect of agitating identical samples on a mechanical "rocker" during the bulk FDA incubation



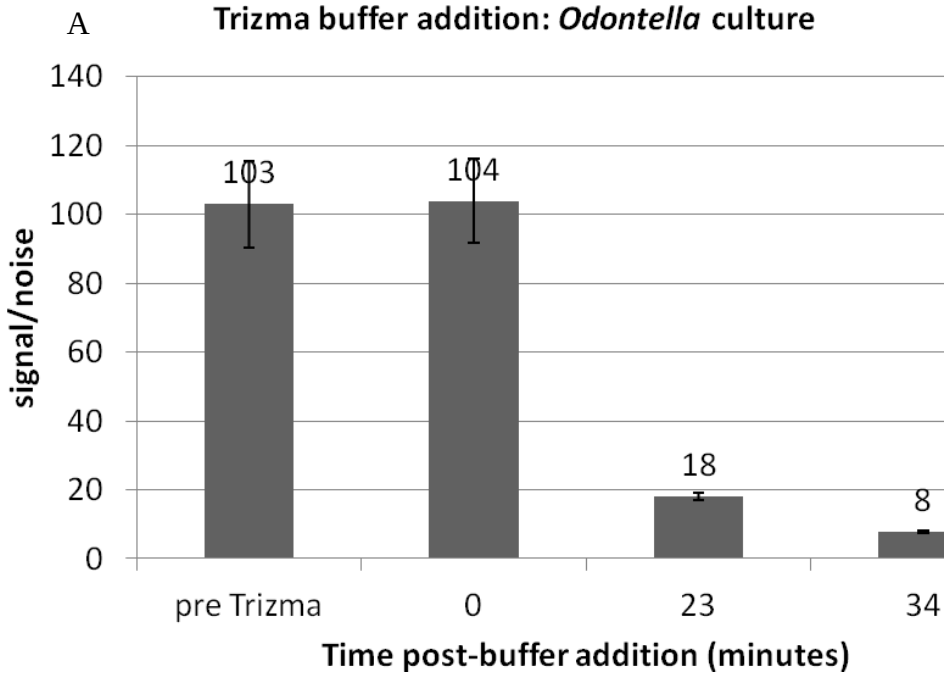
ALKALIZING THE INCUBATION BUFFER TO INCREASE FLUORESCEIN FLUORESCENCE

Fluorescein fluoresces most intensely at or above pH 8.0. Increasing the sensitivity of the assay was a primary goal of the optimization process, therefore it seems logical to measure fluorescence near pH 8. It has been consistently shown, however, that abiotic FP increases drastically with increasing pH, which lead to the selection of pH 6.5 Reagent A as the optimal

incubation buffer. To minimize abiotic FP but increase fluorescein fluorescence, attempts were made to alkalize the incubation buffer above pH 8.0 at the end of the incubation by adding basic reagents. 0.1 N NaOH and the 1M solutions of the basic organic buffers Trizma, Tricine and AMPD were tested. Titration curves were prepared to determine the correct quantity of base to add to Reagent A (pH 6.5) to raise the pH to 8.1. NaOH addition caused complete conversion of all FDA to fluorescein, rendering the fluorescence from all samples and blanks identical. The organic base additions increased fluorescence of both samples and blanks in a proportional manner, resulting in more easily detected fluorescent signals and similar signal/noise ratios relative to non-alkalized samples when the measurement was made immediately following the addition of the base (Figure 20a). Of these alkalizing agents, Trizma proved to yield the highest increase in fluorescein fluorescence and best preservation of signal/noise ratios. Variability among replicate samples did not significantly increase following the addition of the base, suggesting that the increase in fluorescein fluorescence upon the addition of base is consistent and predictable. However, FP from abiotic sources increases markedly after the pH increase, resulting in very high blank fluorescence and an unacceptable reduction in signal/noise ratios if the fluorescent measurement is not made immediately (Figure 20b). To minimize abiotic FP, the addition of Trizma is only recommended under conditions where sensitivity is the primary priority and measurements can be made immediately after the addition of base.

Figure 20: A test of Bulk FDA sample alkalization with Trizma organic base after incubation, before fluorescent measurement a)signal/noise ratio and b) fluorescein fluorescence over time

A



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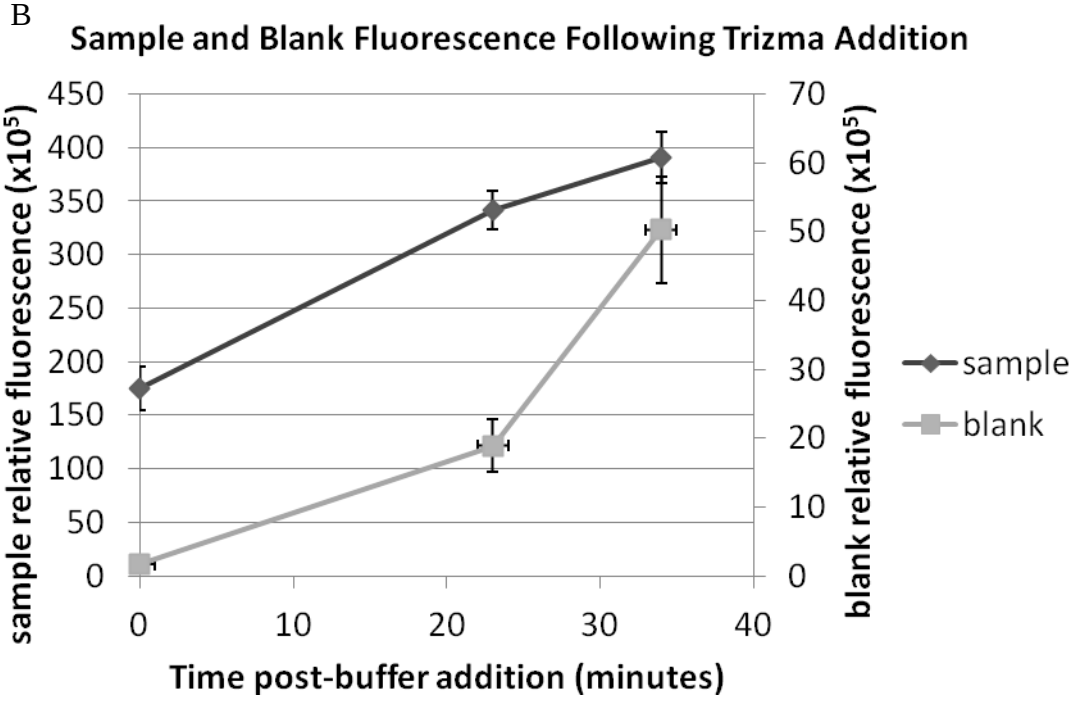


Characterization of the Bulk FDA technique

The fundamental aspect of the Bulk FDA assay, that FP is proportional to living biomass and not dead biomass, is confirmed by experiments presented below involving both cultured organisms and natural samples using the optimized form of the technique. Empirical relationships are elucidated between FP, measured under the standardized, optimized conditions, and other commonly used measures of living biomass.

FLUORESCEIN PRODUCTION FROM STERILIZED SAMPLES

Moss Landing Harbor water was treated with 0 (control), 12, and 60 ppm sodium hypochlorite and extracellular FP was measured at multiple time points during a Bulk FDA incubation (Figure 21). FP proceeds at a constant rate in the untreated sample, while FP is barely detectable above the blank in the treated samples. An experiment testing the effect of duration in the high temperature treatment (70ºC) revealed that increasing incubation time did not affect FP; similar FP was observed in samples held in hot water for 2, 15, 30 and 60 minutes (Figure 22). These results show that reduction of viable biomass by known kill factors results in decreased FP.

Figure 21: The effect of 0 (control), 12, and 60ppm sodium hypochlorite on FP from Moss Landing harbor water

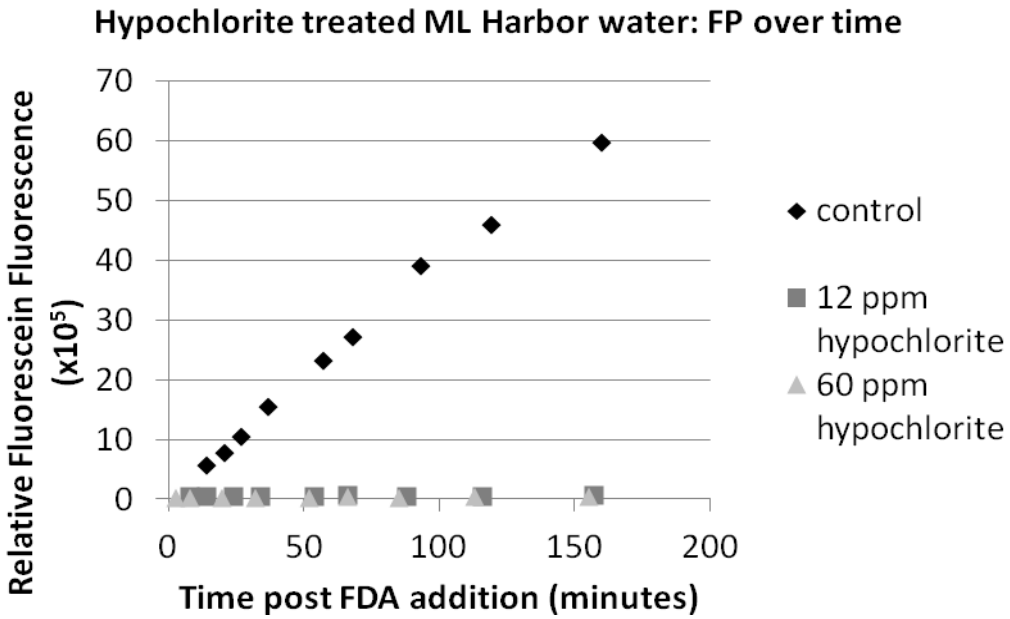
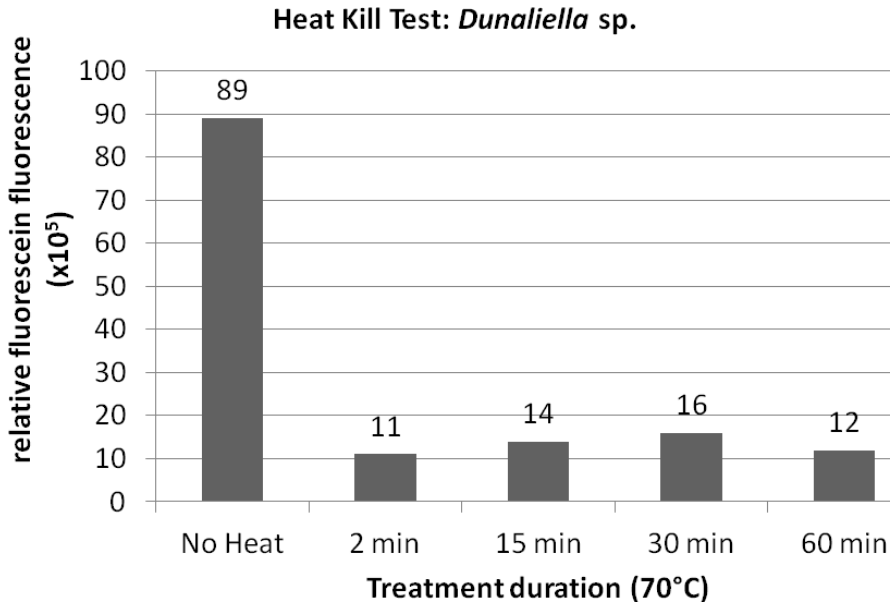


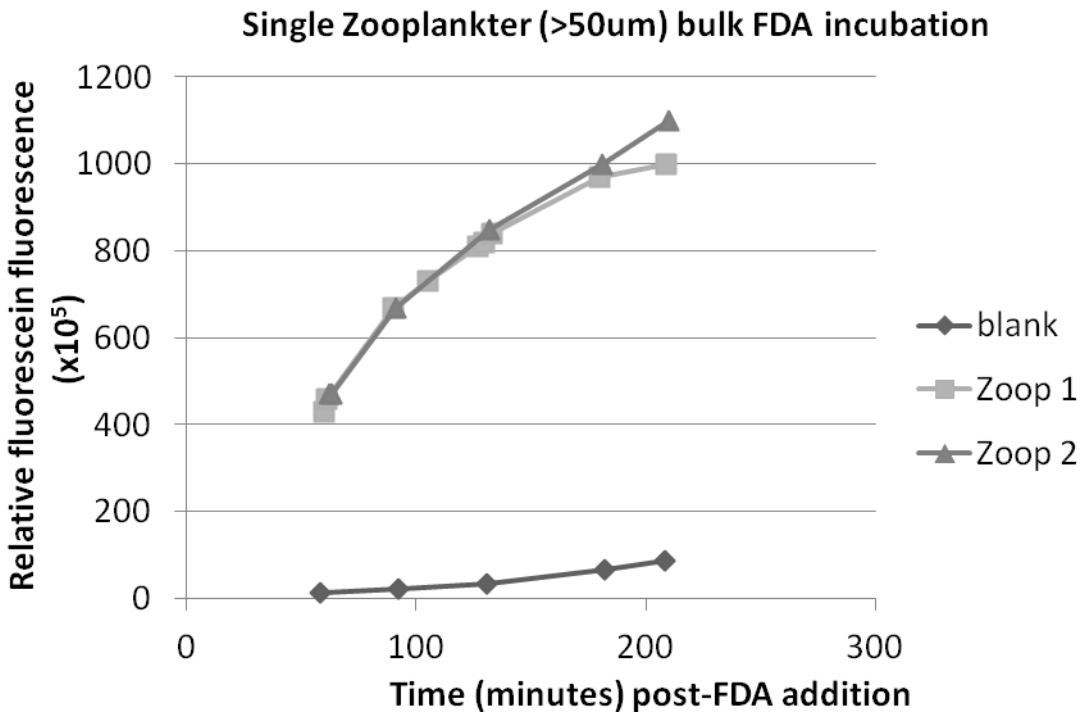
Figure 22: The effect of duration in a hot water bath (70°C) spent by *Dunaleilla* sp. cells prior to being assayed for bulk FDA.



TYPES OF ORGANISMS THAT CAN BE DETECTED

Because esterases are ubiquitous enzymes, the Bulk FDA should, in theory, be able to detect EA from any organism. Most of the optimization tests were performed using algal cultures or natural samples likely to contain phytoplankton and/or microzooplankton, demonstrating the applicability of the assay for these organism types. Additional tests were done to investigate if EA from other types of organisms could be detected. Zooplankton were collected by net tow in Moss Landing Harbor, and individual zooplankters, mostly copepods in nauplii or mature stage, were picked out with forceps, placed in incubation buffer and assayed for Bulk FDA. Fluorescent measurements were made over time after addition of FDA (Figure 23); FP from single zooplankters was 40-fold higher than the blank after a 1-hour incubation, indicating that a single zooplankter produces an easily detected signal with the Bulk FDA technique.

Figure 23: Bulk FDA viability of single copepods



Little attention in this study was given to the assay of terrestrial organisms, however, in one instance, ants and bugs (unidentified) were collected and incubated in the same manner as described for zooplankton above (Figure 24). Both the two ants and single bug showed significantly more FP than the blank after 1-hour. Door handles were swiped around the lab with GF/F filters in an effort to collect bacteria and other microorganisms; the filters were then assayed by Bulk FDA in the usual manner. The signal from swiped filters was variable and slightly higher than the blank in most cases; swiped filters generated signal/blank FP ratios ranging from 1.3- 6.6 (Figure 25).

Marine bacteria were cultured by streaking Moss Landing harbor water onto Difco marine agar plates. The following day, single and multiple colonies were picked off, placed in incubation buffer and assayed for Bulk FDA. Single colonies produced 25x more fluorescence than the blank after a 1-hour incubation (Figure 26). Loading more colonies resulted in proportionally more FP. The number of bacteria cells present in each colony was unknown. The quantity of FP per bacteria cell was investigated more closely

Figure 24: Bulk FDA viability of terrestrial insects were collected outside Moss Landing Marine Laboratories

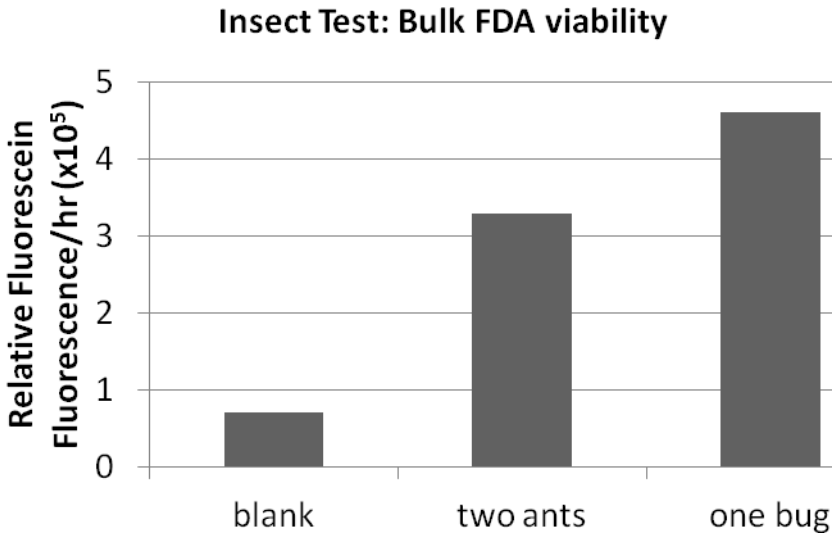


Figure 25: Door handles at Moss Landing Marine Laboratories were swiped with GF/F filters to collect bacterial contamination and were assayed for bulk FDA

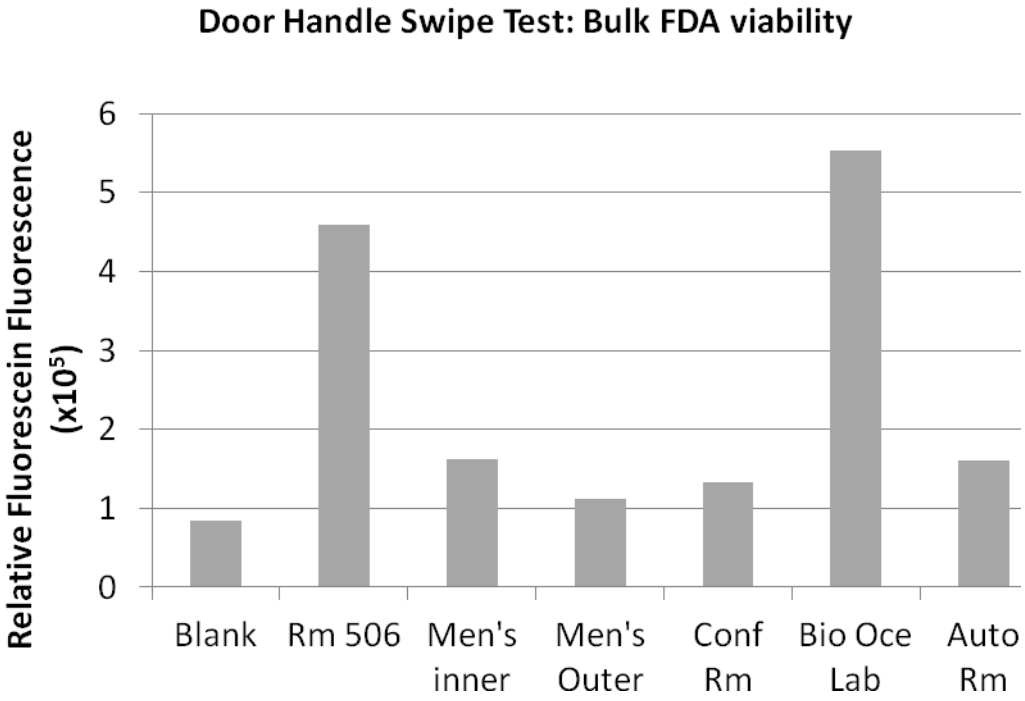
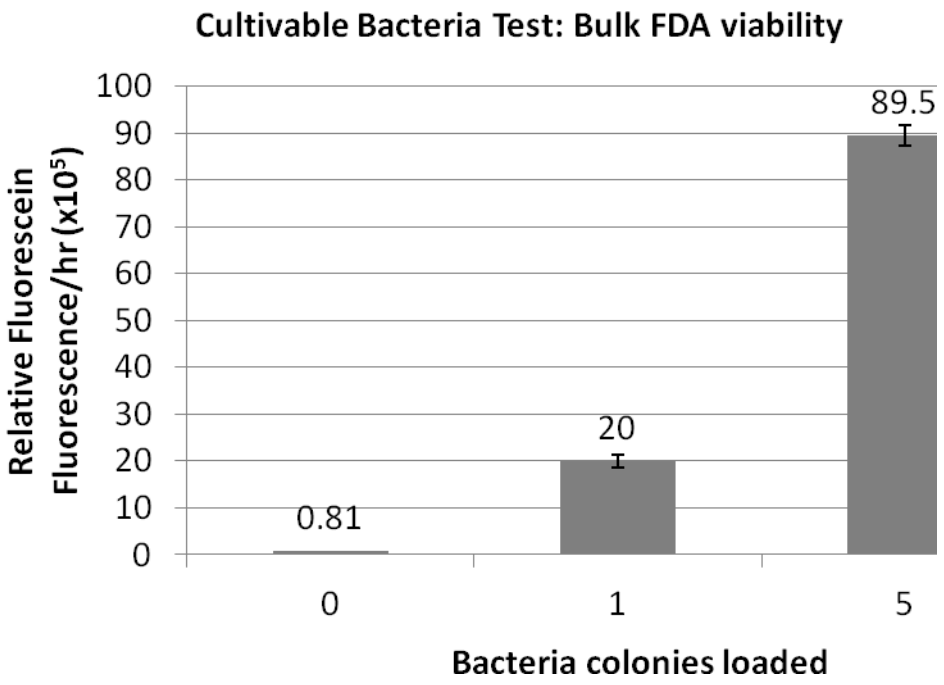
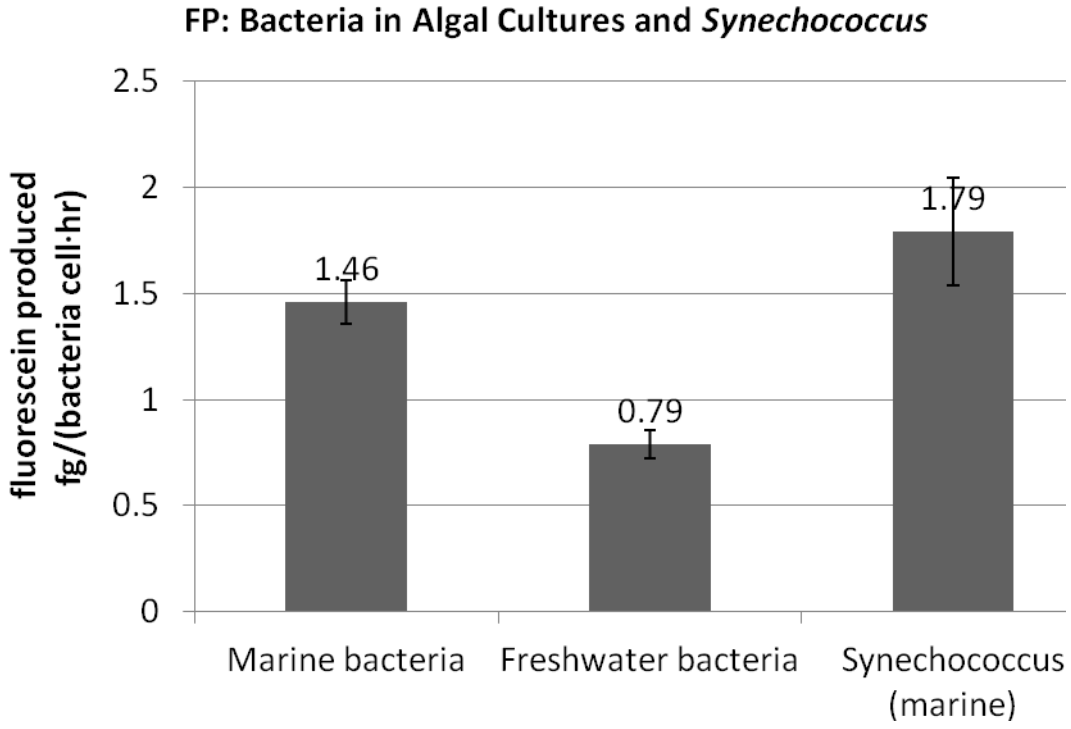


Figure 26: Bulk FDA viability of marine bacteria cultivated on marine agar plates



by utilizing bacteria present in non-axenic algal cultures. Algal cells were removed by filtration from several freshwater and marine algal cultures and the concentration of bacteria was determined using flow cytometry and the green fluorescent DNA stain SYTO-13. Bacteria cells were then captured on GF/F (nominal 0.7µm pore size) filters and assayed for Bulk FDA. It is possible that some smaller bacteria cells could have slipped through the GF/F filter; only bacteria >0.7 µm were assayed. Cultured *Synechococcus* cells, which are photosynthetic marine cyanobacteria 1.7µm in diameter, were counted by flow cytometry and assayed in the same manner. Using the assumption that all the bacteria which contributed towards the flow cytometric count were collected on the GF/F, marine and freshwater bacteria FP was equal to 1.46 and 0.79 fg/cell.hr, respectively, while *Synechococcus* FP was equal to 1.79 fg/cell.hr (Figure 27).

Figure 27: Fluorescein production rates per cell of marine and freshwater bacteria isolated from non-axenic algal cultures and of the cultured cyanobacteria *Synechococcus*

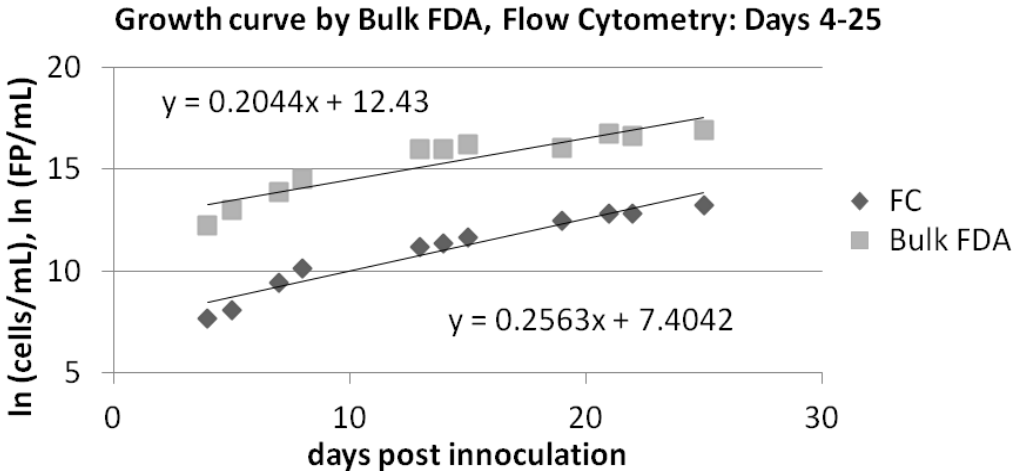


GROWTH EXPERIMENT

An experiment was designed to test if the Bulk FDA technique could measure the growth rate of an algal batch culture. This experiment also investigated if FP per cell changed as the culture grew. A large volume (2L) batch culture of the unicellular alga *Tetraselmis* sp. was inoculated and monitored over a period of 36 days. Every few days the culture was mixed and a small volume was removed to measure the bulk biomass indicator, FP, and the numeric concentration of live cells by flow cytometry (FC). Measurements of both parameters over time were plotted as growth curves in which the natural logarithm of the live indicator per unit volume (i.e. live cells/mL or FP/mL filtered) was plotted against the days after inoculation; the slope of the linear regression of these plots is the effective growth rate, μ (Figure 28a). Between Day 25 and 36, the cell concentration actually decreased, therefore, Day 36 is not included in the growth

Figure 28: The growth rate of a batch *Tetraselmis* sp. culture measured by flow cytometry (numeric cell concentration) and bulk FDA (bulk biomass indicator) over the a) entire growth period, b) first 15 days, c) and last 10 days

A



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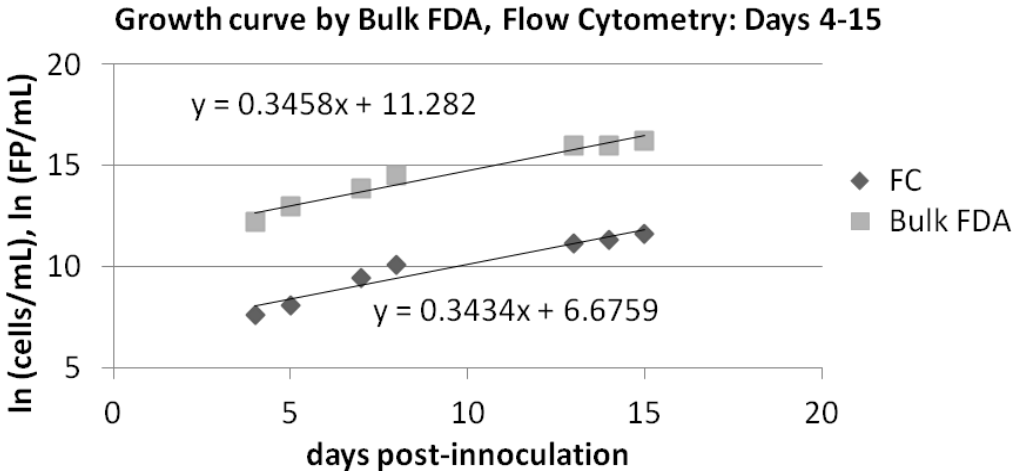

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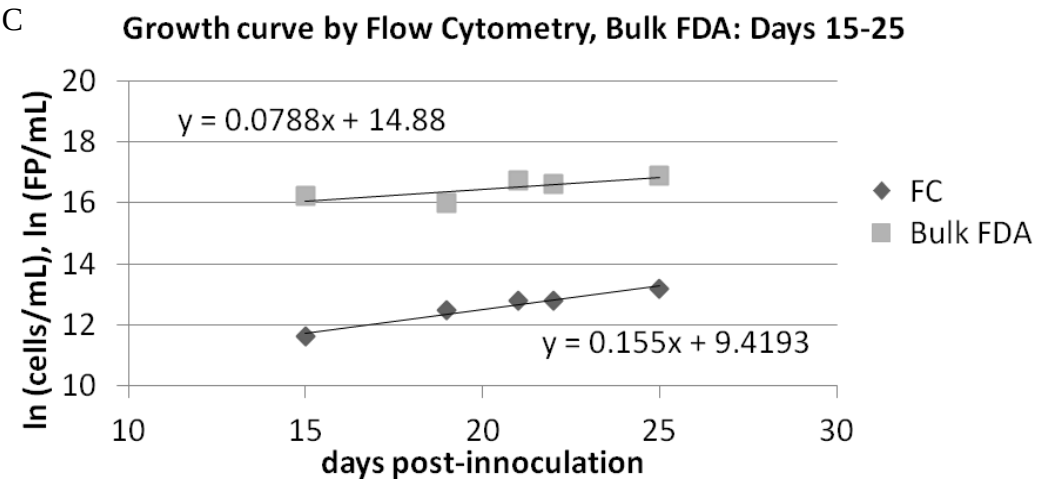


plots. Over the 25 days of growth, the FC generated μ=0.256 while the Bulk FDA μ=0.204.

Breaking the plot into two sections provides insight into this discrepancy. From Days 4-15 the two methods provide nearly identical measurements of growth rate (FC μ=0.343, Bulk FDA μ=0.346) (Figure 28b). From Day 15-25 the FC growth rate is nearly double the Bulk FDA growth rate (FC μ=0.155, FP μ=0.079) (Figure 28c). Clearly, the cells are growing more slowly during this second phase of the experiment, and slowing of the growth rate is more pronounced when measured by Bulk FDA.

The difference between the growth rate estimated by FC and by Bulk FDA during days 15-25 of growth can be explained by investigating the FP/cell ratios over time (Figure 29). The FP/cell over the first 15 days after inoculation is variable but without an evident trend, as individual values range from 1.78-2.99 pg/cell.hr. From days 19-36, the FP/cell drops considerably and does not recover, ranging from 0.58-1.11 pg/cell.hr. During this second phase of the batch culture, the decrease in FP/cell ratios leads to lower estimates of growth rate by Bulk FDA relative to the numeric count. The decrease in FP/cell observed over time was not due to a decrease in cell volume, as the size of cells, measured by the forward scatter detector of the flow cytometer, did not change significantly during the growth period (Figure 30). These results show that the FP/cell of a single species can vary by 5-fold depending on the ambient conditions. Nutrient deprivation is likely to be a main cause of the observed decrease in FP/cell, while it is possible that self-shading or some unknown factor could contribute as well.

Figure 29: The FP/cell rate of a *Tetraselmis* sp. batch culture over time.

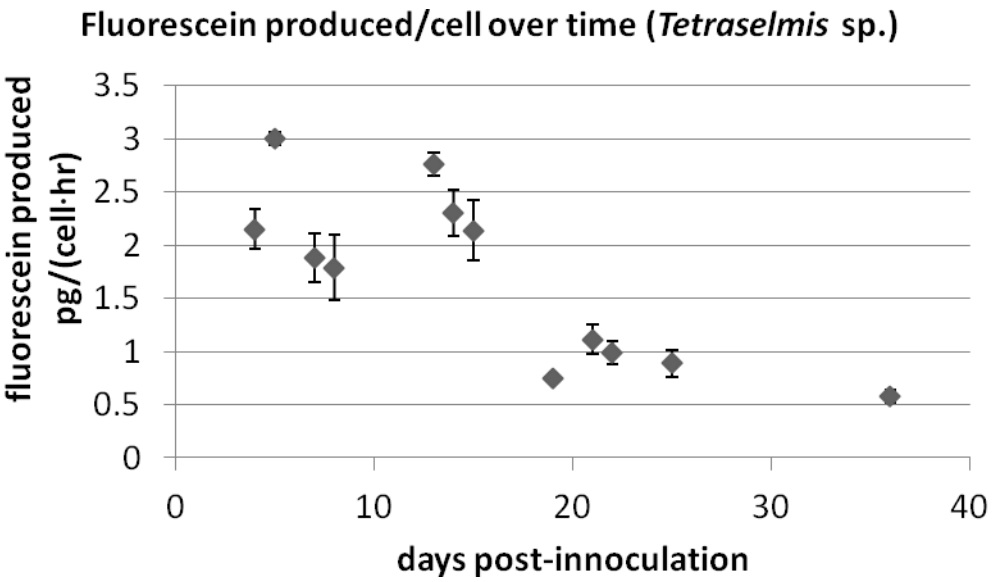
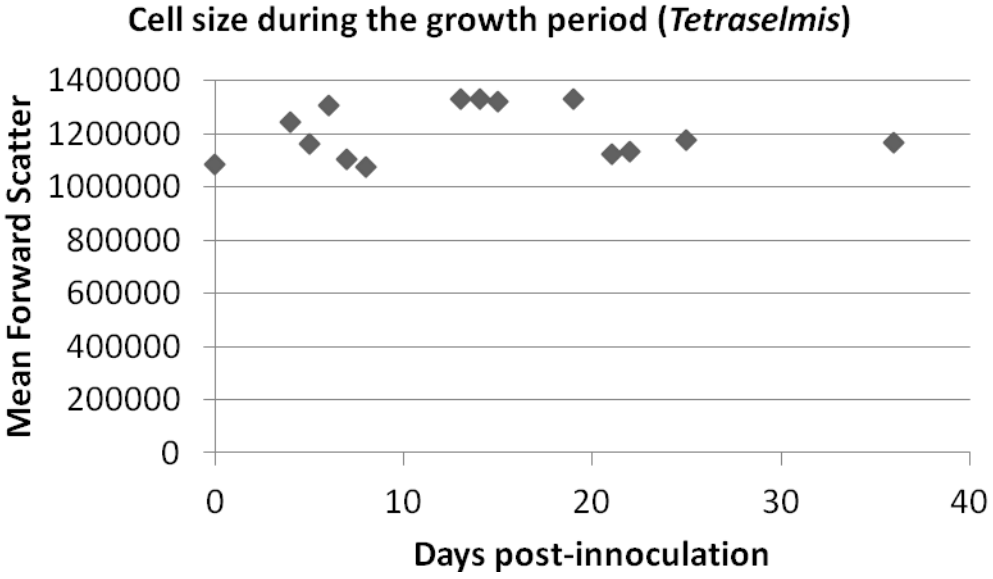


Figure 30: The relative cell size of a *Tetraselmis* sp. batch culture over time, given as the mean forward scatter signal of an Accuri C6 flow cytometer.



MONTEREY BAY DATASET: BULK FDA, ATP, LIVE CELL CONCENTRATION

Correlation of fluorescein production with ATP

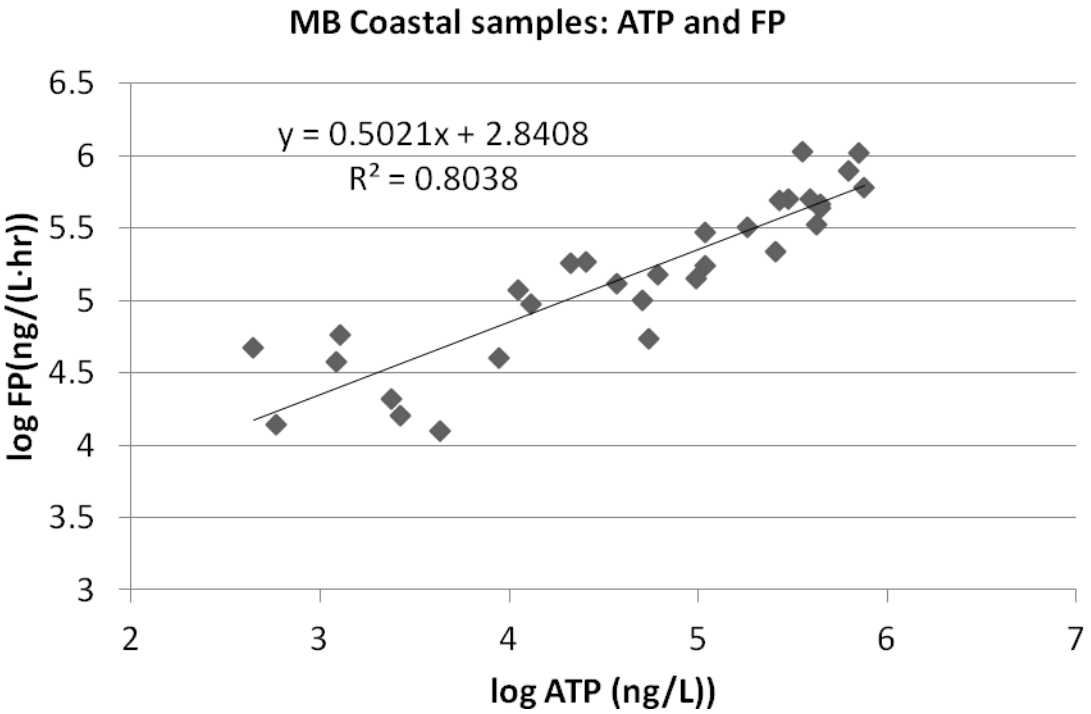
Natural samples were collected along the coast in Pacific Grove, Monterey and Moss Landing, as well as from Moss Landing harbor. Some samples were manipulated to reduce or increase ambient viable biomass. Sodium hypochlorite was used at several concentrations to reduce viable biomass. Samples were treated with Guillard's marine enrichment solution and incubated in a temperature controlled illuminated incubator (15°C) to increase viable biomass. Controls for nutrient enriched samples included a sample placed in the illuminated incubator without nutrients and a sample in the dark without nutrients. The nutrient enrichment treatment also serves to mimic nutrient variability expected in nature, such as a pulse of nutrients from upwelling or terrestrial sources. FP, ATP and live cell concentration (10-50 micron ESD) by FDA flow cytometry were measured on each of the coastal samples.

Log-log (base 10) plots were made to establish the relationship between ATP and FDA, as the data spanned several orders of magnitude. Linear regression of log(ATP) vs. log(FP) values from this sample set reveals a positive, relatively constant relationship with the following equation (Figure 31):

$$\log\left(\mathrm{FP}\left(\frac{\mathrm{ng}}{\mathrm{L.hr}}\right)\right) = 0.50 * \log\left(\mathrm{ATP}\left(\frac{\mathrm{ng}}{\mathrm{L}}\right)\right) + 2.8 \quad (R^2 = 0.80)$$

The slope <1 of the above regression reveals that ATP data span a greater range than FP. ATP ranges over slightly more than 3 orders of magnitude, while FP values range over 2 orders of magnitude. Non-manipulated samples showed FP (ng/L.hr)/ATP (ng/L) ratios between 0.74-7.24 with a mean of 1.87. Samples manipulated with low levels (0.12-6 ppm) of hypochlorite showed slightly higher FP/ATP ratios (range 1.11-10.7, mean = 3.28), as the reduction in ATP was more pronounced than in FP. The samples treated with high bleach concentration (60 ppm) showed disparity between the decrease in ATP and in FP, which results in the highest ratio (FP/ATP) values and highest variance observed in this study (range 4.7-107, mean 35.8). The nutrient enriched samples showed proportional increases in ATP and FP, which results in FP/ATP ratios (mean 1.8) similar to the non-manipulated samples.

Figure 31: The relationship between FP and ATP from manipulated and non-manipulated coastal samples

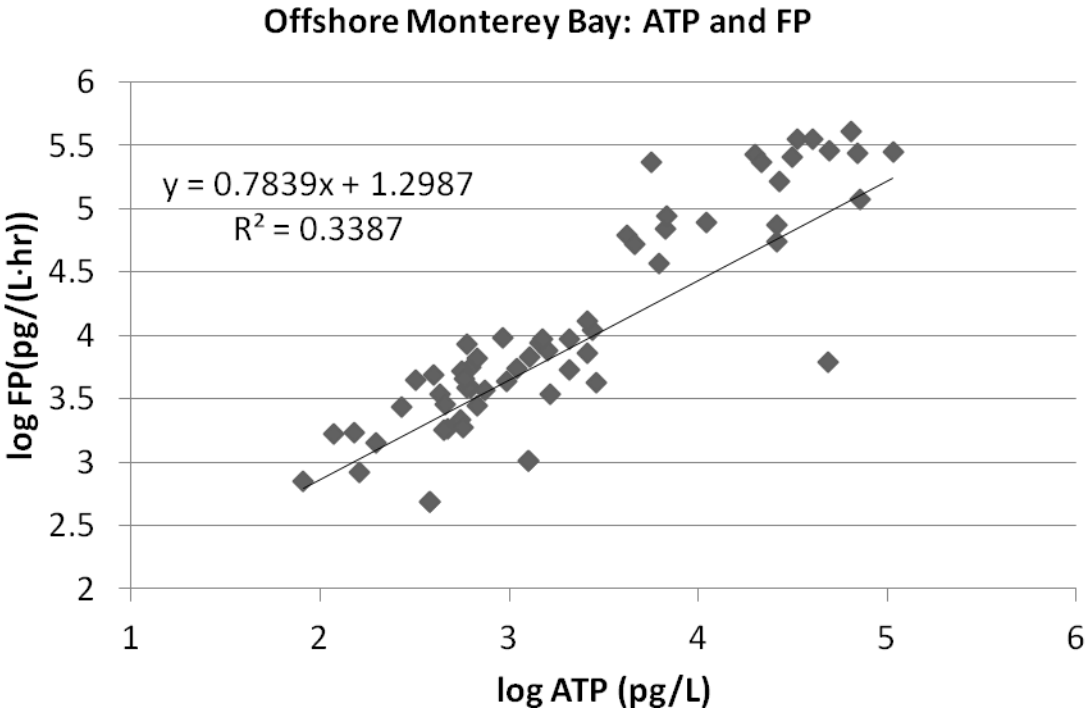


Additional samples were collected aboard the Research Vessel *Point Sur* at several sites offshore in Monterey Bay on March 28-29th, 2012 to further investigate the relationship between ATP and Bulk FDA. Samples were taken at multiple depths in the water column (surface to 4000 m) using a CTD rosette, and processed for ATP and FP shortly after collection. The relationship is again relatively consistent and the plot of log(ATP) vs. log(FP) reveals the following equation (Figure 32):

$$\log\left(\mathrm{FP}\left(\frac{\mathrm{ng}}{\mathrm{L.hr}}\right)\right) = 0.78 * \log\left(\mathrm{ATP}\left(\frac{\mathrm{ng}}{\mathrm{L}}\right)\right) + 1.3 \quad (R^2 = 0.34)$$

Again, the ATP dataset ranges over 4 orders of magnitude, while the Bulk FDA values only range over 3 orders of magnitude, resulting in a slope on the log-log plot < 1. FP (ng/L.hr)/ATP (ng/L) ratios were variable among these samples, ranging from 0.82-41. However, no trend was observed in the FP/ATP ratio with depth.

Figure 32: The relationship between FP and ATP from water column profile samples (surface-4000m) collected in Monterey Bay, March 2012



Correlation of fluorescein production with numeric concentration of live cells 10-50 µm

The relationship among coastal samples between FP and live cell concentration measured by flow cytometry is positive and relatively constant. Linear regression of the log-log plot between FP and live cell concentration (both manipulated and non-manipulated samples) reveals the following equation (Figure 33):

$$\log\left(\mathrm{FP}\left(\frac{\mathrm{pg}}{\mathrm{hr}}\right)\right) = 0.51 * \log(\text{live cells } (10-50\mu\mathrm{m})) + 2.8 \quad (R^2 = 0.50)$$

The slope <1 of the above log-log regression reveals that live cell concentration varies over a greater range than FP.

The empirical measurements of FP and live cell concentration allow one to estimate a per cell (10-50 µm ESD) FP rate. A histogram (Figure 34) shows the frequency of FP/cell values observed among these samples; FP/cell values in the range 0-

1 pg/cell.hr were the most commonly observed. Non-manipulated samples showed FP (pg/cell.hr) values between 0.37-18.0 with a mean of 2.70. Samples manipulated with low levels (0.12-6 ppm) of hypochlorite showed elevated FP (pg/cell.hr) values (range 0.4-19.4, mean = 6.32) relative to non-manipulated samples, as live cell concentration decreased more drastically than FP. Samples treated with high bleach concentration (60 ppm) all scored 0 live cells according to the cytometric technique, prohibiting one from calculating the per cell FP. The nutrient enriched samples and illuminated controls showed greater increases in FP than in live cell concentration, resulting in FP (pg/cell.hr) values of 1.44 and 1.22 respectively, which were higher than the FP (pg/cell.hr) of the dark, non-enriched control (1.19). These results suggest that the per cell FP increases upon the addition of light and increases most drastically when light and nutrients are provided.

Figure 33: The relationship between FP and live cell concentration measured by flow cytometry from coastal Monterey Bay samples (10-50µm size class)

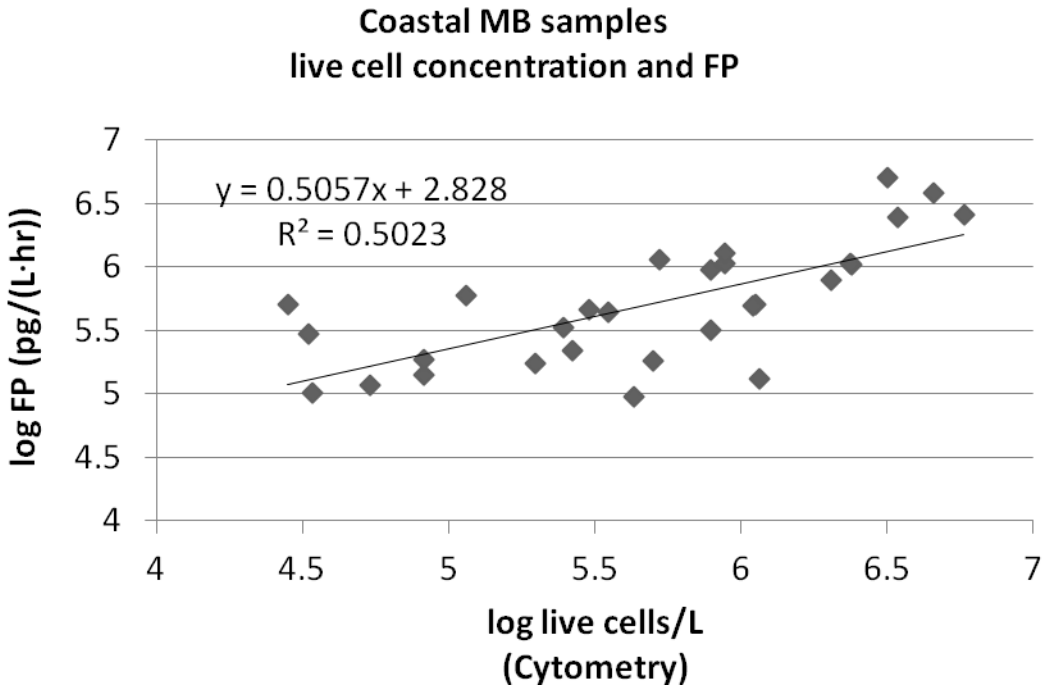
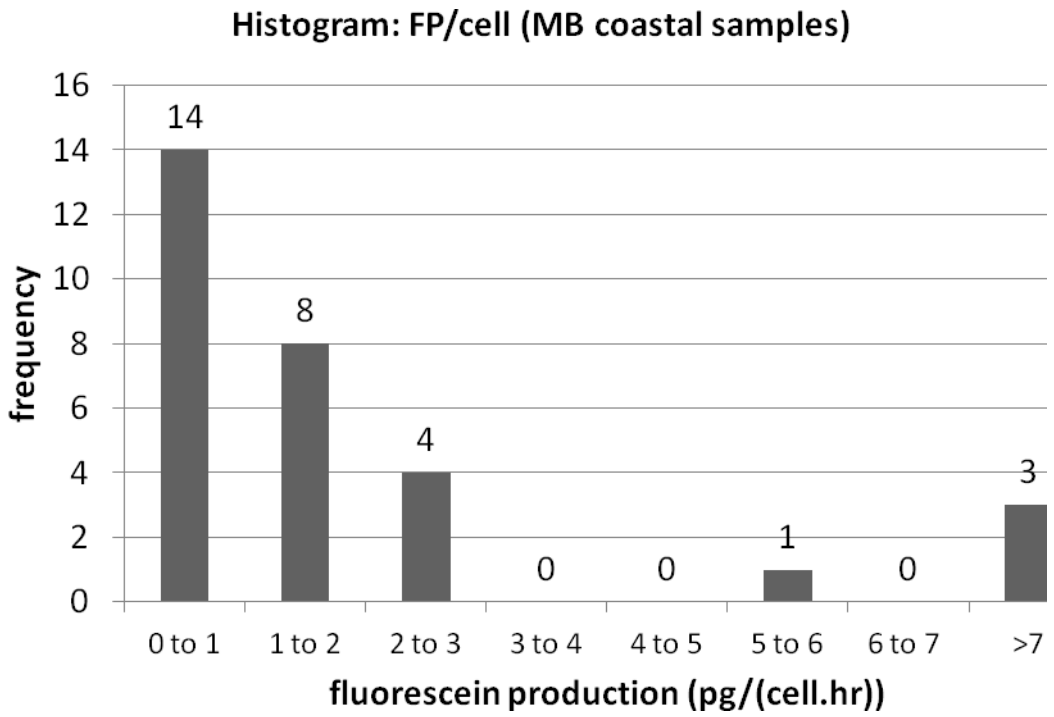


Figure 34: Histogram of FP/10-50µm cell ratios observed among coastal Monterey Bay samples



DETERMINATION OF THE RELATIONSHIP BETWEEN CELL VOLUME, FLUORESCEIN PRODUCTION AND ATP USING ALGAL CULTURES

In order to determine the FP/cell rate from unicellular algal cultures of known cell volume, an experiment was performed in which FP, cell concentration, ATP, and cell volume were measured simultaneously from all freshwater and marine algal cultures cultivated in the laboratory (listed in Table 1). The resulting dataset was analyzed using linear regression between the measured parameters. To accommodate the large ranges in biomass measured, the logarithm (base 10) of each parameter was taken and used to create plots for regression.

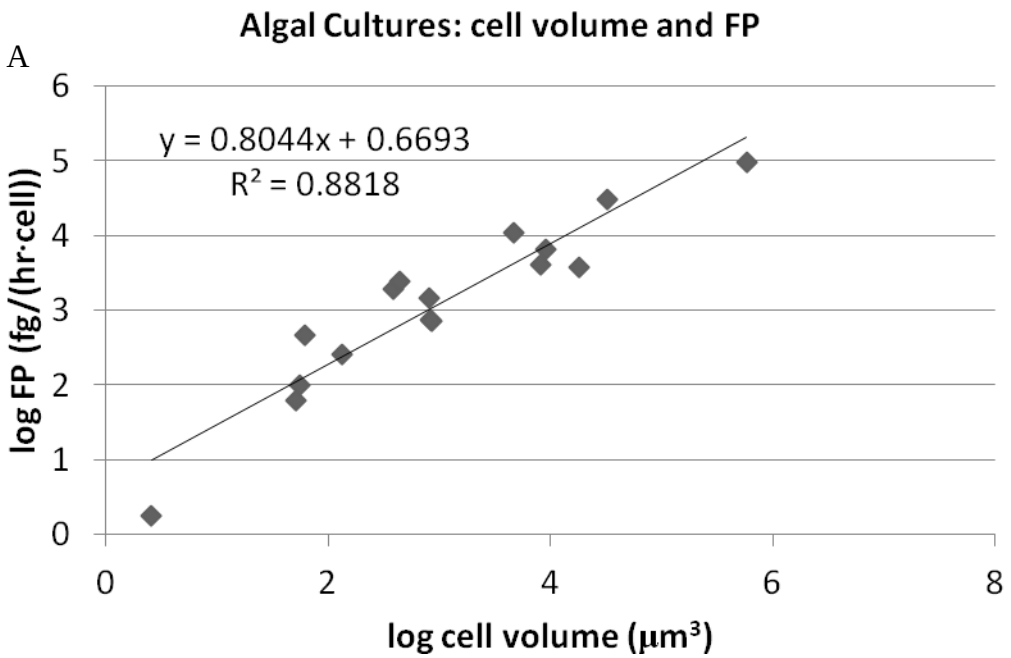
Fluorescein production and cell volume

Linear regression of log(cell volume) and log(FP) reveals the following equation:

$$\log\left(\mathrm{FP}\left(\frac{\mathrm{pg}}{\mathrm{hr.\,cell}}\right)\right) = 0.80 * \log\left(\frac{\text{cell volume}(\mu\mathrm{m}^3)}{\text{cell}}\right) + 0.67 \quad (R^2 = 0.88)$$

Although variation exists among the strains tested, it is clear that a significant and positive relationship exists between FP and cell volume (Figure 35a). The slope <1 on the log-log plot shows that cell volume varies over a wider range than FP. FP/µm^3 ratios are lower for the largest cultures, causing the FP values to span over a narrower range than cell volume. Individual FP/µm^3 ratios ranged from 0.12 - 7.48 fg/hr.µm^3 for *Coscinodiscus* (cell volume = 792,000µm^3) and *Chlorella* (cell volume = 62µm^3), respectively. A plot of FP/µm^3 ratios vs. cell volume, Figure 35b, shows a trend of declining FP/µm^3 ratios with increasing cell volume. No clear trend was observed between freshwater and marine cultures; both types of algae showed comparable FP/µm^3 ratios, which suggests that the EA of both freshwater and marine phytoplankton are similarly affected by the buffer.

Figure 35: a) The relationship between cell volume and fluorescein production per cell per hour of each cultured alga b) the relationship between FP/µm^3 and cell volume



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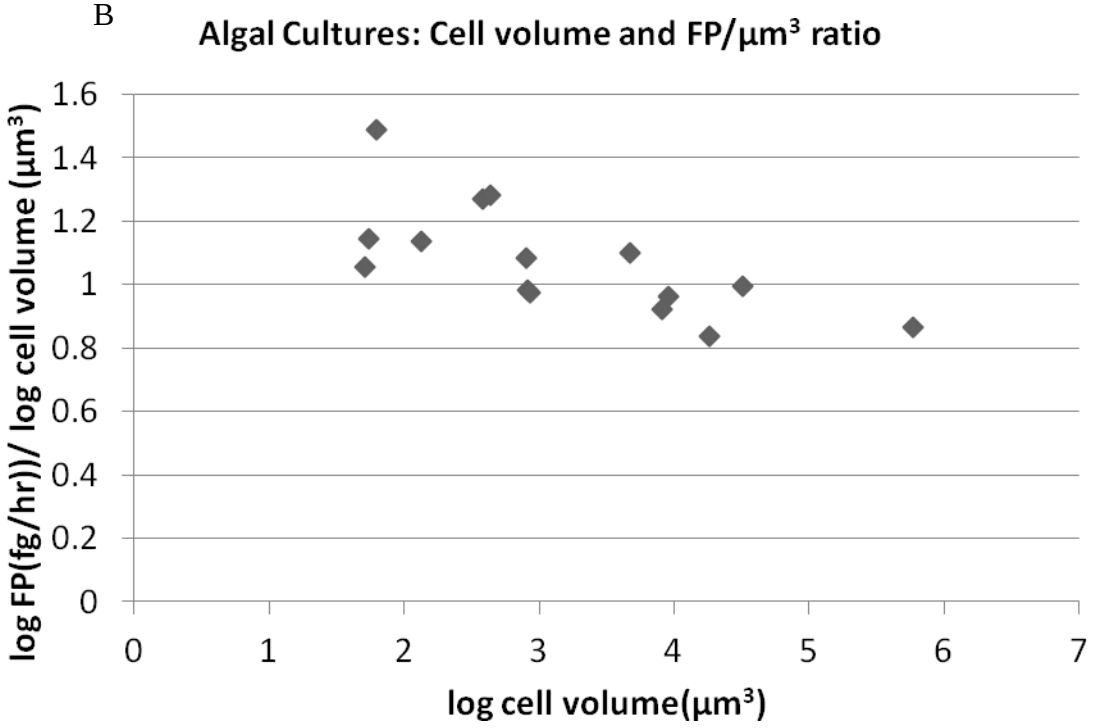


ATP and cell volume

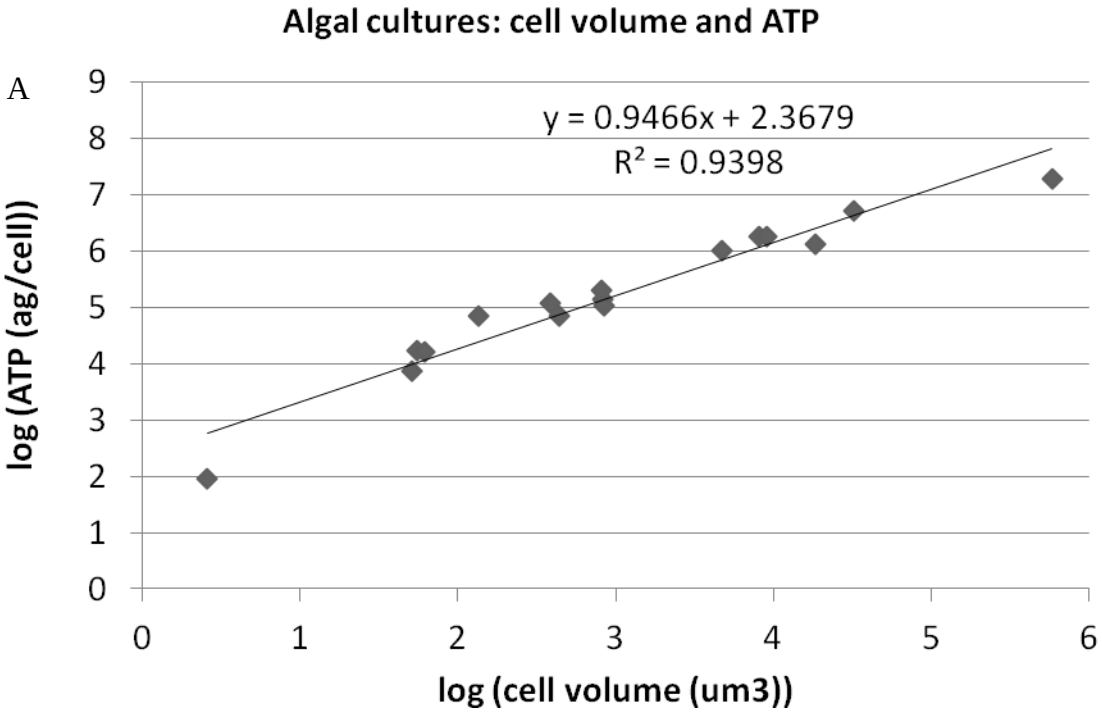
Linear regression of log(ATP) and log(cell volume) reveals the following equation:

$$\log\left(\mathrm{ATP}\left(\frac{\mathrm{ag}}{\mathrm{cell}}\right)\right) = 0.93 * \log\left(\frac{\text{cell volume } (\mu m^3)}{\mathrm{cell}}\right) + 2.41 \ (R^2 = 0.93)$$

A significant and positive relationship clearly exists between the ATP and cell volume (Figure 36a). The slope <1 on the log-log plot shows that cell volume does vary over a wider range than ATP, but to a lesser extent than FP and cell volume. The R^2 value is also higher than in the cell volume vs. FP regression; these results show that ATP correlates more closely with cell volume than FP does. Individual ATP/µm^3 ratios were less variable than FP/µm^3 ratios, ranging from 25 - 517 pg/µm^3 for *Coscinodiscus* (792,253µm^3) and *Haematococcus* (134µm^3), respectively. A plot of cell volume vs. ATP/µm^3 ratios, Figure 36b, reveals a trend of decreasing ATP/µm^3 ratios with increasing cell volume.

Figure 36: a) The relationship between cell volume and ATP concentration for each cultured alga b) the relationship between ATP/µm^3 and cell volume

A



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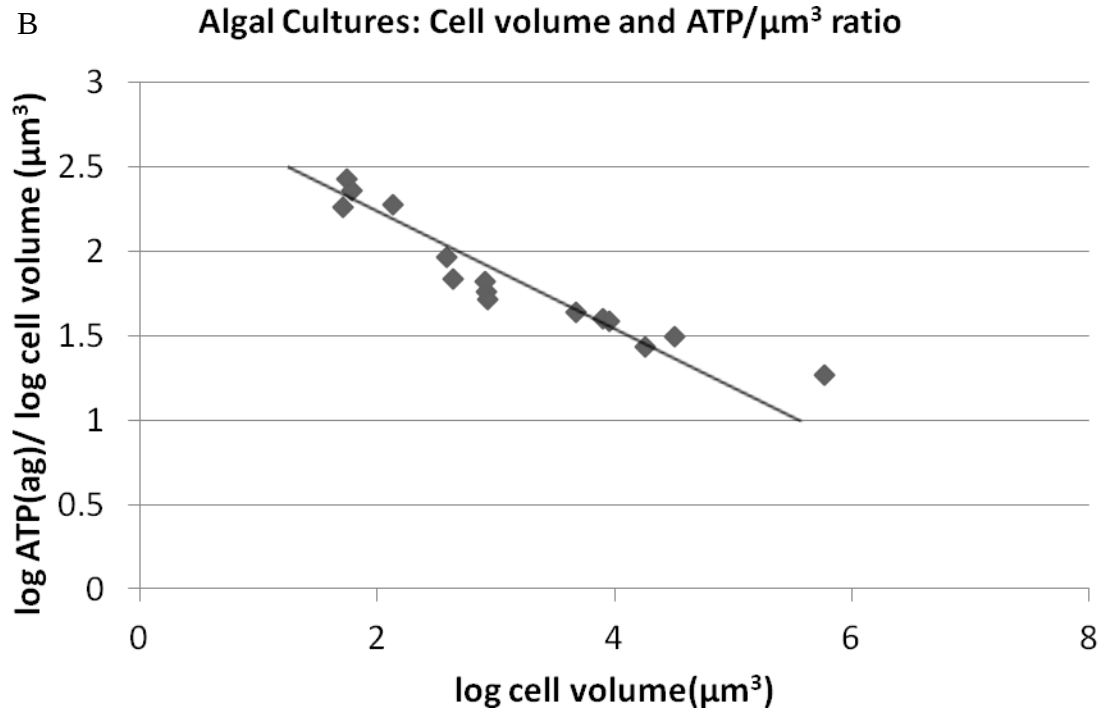


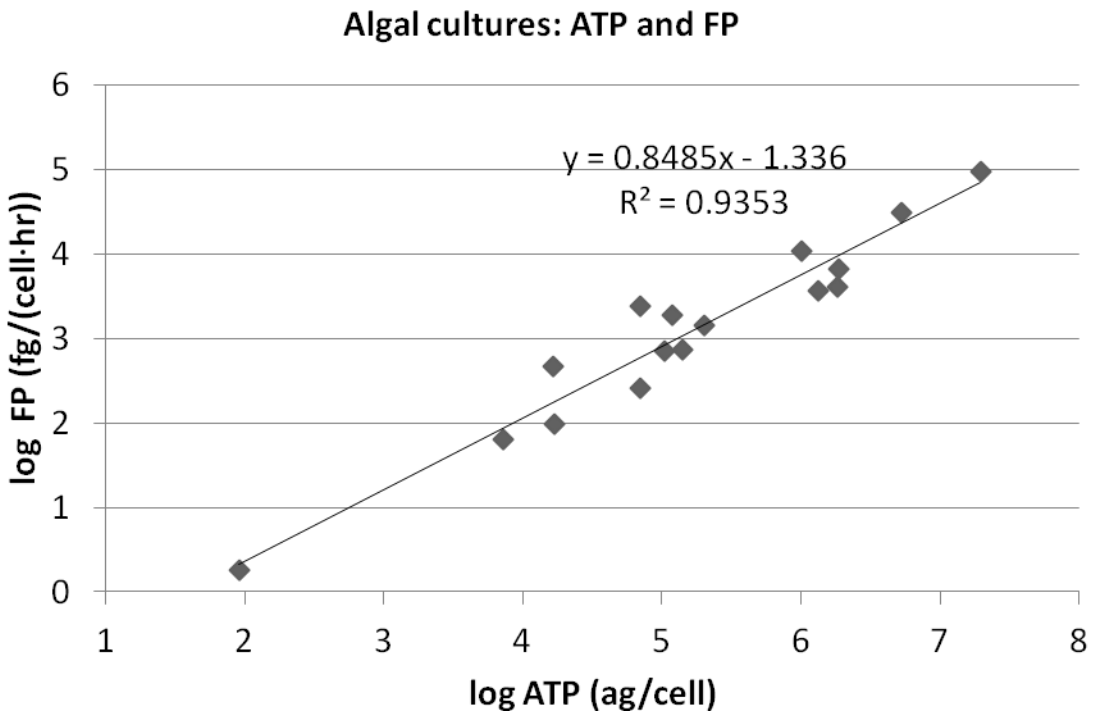
Fluorescein production and ATP

Linear regression of log(FP) and log(ATP) reveals the following equation:

$$\log\left(\mathrm{FP}\left(\frac{\mathrm{pg}}{\mathrm{hr.\,cell}}\right)\right) = 0.85 * \log\left(\frac{\mathrm{ATP(ag)}}{\mathrm{cell}}\right) - 1.34 (\mathrm{R}^2 = 0.94)$$

As expected, a significant and positive relationship exists between FP and ATP (Figure 37). The slope <1 on the log-log plot shows that ATP varies over a wider range than FP, which was expected based on the relationships that FP and ATP show with cell volume. Individual FP/ATP ratios ranged from 2.2 - 34 (fg FP/hr)/(fg ATP) for *Helicotheca tamensis* (8,064µm^3) and *Dunaliella* sp. (434µm^3), respectively. Chlorophytes generally show higher FP/ATP ratios (including *Dunaliella*, *Tetraselmis, Chlorella, Staurastrum*), while other taxa, mostly diatoms, show lower FP/ATP ratios. No significant difference was observed in the FP/ATP ratios between freshwater and marine algal cultures.

Figure 37: The relationship between ATP/cell and FP/cell.hr for each cultured alga.



Method detection limit and precision of the Bulk FDA technique

The method detection limit (MDL) of the Bulk FDA assay was determined by following the protocol outlined by Skoog, et al in his book, *Principles of Instrumental Analysis.* Approximately 6,000 *Tetraselmis* sp. cells were filtered onto GF/F filters (n=9) and processed for Bulk FDA using the optimized protocol (n=9). Samples were measured on the high sensitivity SPEX fluorometer and handheld Aquafluor fluorometer. Cell-free blanks were included and used to correct sample fluorescence. The standard deviation of samples, in units of fluorescein concentration, was 0.09 and 0.30 μg/L on the SPEX and Aquafluor fluorometer, respectively. These standard deviations are multiplied by the 1-sided t-value for 8 degrees of freedom (2.896) to yield the MDLs of 0.26 and 0.87μg/L fluorescein on the SPEX and Aquafluor fluorometers, respectively. 2.5mL incubation volume is typically used; multiplying each MDL by the incubation volume reveals that 648 and 2,166pg of FP can be detected by the SPEX and Aquafluor fluorometers, respectively. If we consider, for example, that 15μm is the most likely mean cell size of a 10-50μm ballast water sample, and we know the expected FP rate by a 15μm cell is 1.91 pg/cell.hr, we calculate that 339 and 1,134 cells must be harvested to reach the method detection limit when using the SPEX and Aquafluor fluorometers, respectively.

The datasets in which ATP and Bulk FDA were measured from identical samples in replicates, including algal cultures, coastal Monterey Bay samples and ballast water samples, were used to compare the precision of the two assays (Table 3). The precision of the Bulk FDA technique, calculated as the coefficient of variation (CV):

$$CV(\%) = \left(\frac{SD}{mean} \right) * 100\%$$

was higher than the extraction and measurement of ATP for all 3 datasets. The Bulk FDA assay typically has a coefficient of variation (CV) ≤ 10%, however, including treated and therefore low biomass samples as in the coastal and ballast samples results in CVs >10%.

Table 3: The precision of the Bulk FDA and ATP assays observed from 3 distinct sample sets

Sample set	n	Bulk FDA CV (%)	ATP CV(%)
Algal cultures	4	**5%**	**14%**
Monterey Bay coastal samples	3	**9%**	**17%**
Ballast samples	3	**23%**	**27%**

DISCUSSION

Fluorescein Budget: Intracellular vs. Extracellular

In Bulk FDA incubations with cultured algal cells, roughly 97% of the total fluorescein produced (intracellular + extracellular) is present in the extracellular solution after a 1-hour incubation. However, several factors could cause this estimation of intracellular and extracellular fractions to be inaccurate. It is possible that more fluorescein is present in the intracellular fraction than estimated above, but that the measurement of fluorescein is more efficient in the extracellular medium than inside cells, resulting in an underestimation of the relative proportion of intracellular fluorescein. The cell wall, plasma membrane or other cellular structures could optically interfere with the measurement of intracellular fluorescein fluorescence. It is also possible that mechanical stress caused by the processes to remove cells, centrifugation or filtration, could cause cells to release some or all of their intracellular fluorescein into the extracellular solution, resulting in an underestimation of the intracellular fraction. Conversely, the intracellular fraction could be overestimated if the cytoplasmic pH exceeds that of the extracellular buffer (pH 6.5), as fluorescein fluoresces more intensely as pH increases. In either case, it is more sensitive and reproducible to measure extracellular fluorescein, thereby eliminating any variability caused by the intracellular fraction, and the technique has been devised to measure fluorescein in Reagent A buffer.

pH and abiotic fluorescein production

During the optimization experiments, it became clear that pH is important for many aspects of the Bulk FDA assay. Fluorescein fluorescence is very dependent on pH, the fluorescence of a single standard ranges 56 times between pH 3.9 and 8. This relationship gives the user power to increase or decrease the fluorescence of a sample or standard by adjusting pH. A primary goal of the optimization process is increasing sensitivity; the usefulness of the assay is proportional to its ability to reliably measure low biomass levels. Therefore, it is logical to measure the fluorescent result of the assay at the optimum pH for fluorescein fluorescence. However, incubating a sample at high

pH has drawbacks that counteract sensitivity gains in the intensity of fluorescein fluorescence.

High abiotic FP was observed in the majority of incubation buffers tested here. Clarke et al. 2001, when attempting to devise a protocol for measuring bulk microbial activity in commercial products using FDA, observed high amounts of abiotic FP in all of the incubation buffers and bacterial growth medium tested, and concluded that no acceptable incubation buffer had currently been found. The results presented here show that abiotic FP is correlated with pH to an even greater extent than fluorescein fluorescence. The increased efficiency of fluorescein fluorescence at elevated pH does contribute towards the increase in abiotic FP, however, abiotic FP increases to a greater extent than could be due to the increased efficiency of fluorescein fluorescence alone. Between pH 7 and 8, for example, fluorescein fluorescence increases by 1.3-times, while abiotic FP from an incubation with FDA increases by 13-times. When using incubation buffers above pH 7, this increase in abiotic FP results in reduced signal/noise ratios, as abiotic FP increases more drastically than biological FP between pH 7-8. When using incubation buffers between pH 6 and 7, both blank and sample fluorescence increase proportionally, resulting in signal/noise ratios that are similar within this range.

In an effort to minimize abiotic FP while maximizing the sensitivity of the assay, it was proposed that the sample could be alkalized at the end of the incubation and immediately before making the fluorescent measurement. Approximate 1.6-times increases in the sample and blank fluorescence were observed immediately after adding the base, which raised the pH from 6.5 to 8.1. This step can be useful for users demanding greater sensitivity, such as those using an insensitive fluorometer. Care must be taken, however, to make this measurement as soon as possible after adding the base to avoid false positives, as the rate of FP increases rapidly after the addition of base. If the measurement is not made immediately, samples without any viable biomass, such as a cell-free blank or successfully sterilized sample, will show significant rates of FP, which could lead to the conclusion that the sample contained viable biomass. Therefore, alkalization of samples is not recommended for typical execution of the Bulk FDA assay.

Temperature and the Bulk FDA assay

The Bulk FDA assay is an indicator of enzyme activity, therefore, it should respond to changes in temperature like kinetic enzyme models predict. The Q_{10} coefficient for enzyme catalyzed reactions, such as FDA hydrolysis, is typically 2-3 (Quinlan 1980). Rotman and Papermaster (1965), while studying the hydrolysis of FDA by mammalian cells, found that Q_{10} =1.78 for the intracellular accumulation of fluorescein. The Q_{10} coefficient observed in this study for extracellular FP by *Tetraselmis* sp. (2.05) is well within the expected range. The enzymes hydrolyzing FDA perform as expected in response to temperature, which suggests that extracellular FP is a direct indicator of cellular EA and the dependence of the Bulk FDA assay on FDA influx and fluorescein leakage through cell membranes does not compromise this relationship. This finding also implies that the esterases are not denatured by the pH or temperature present during the incubation. The predictable response of FP with temperature greatly improves the convenience of the assay; it can be performed at any temperature likely to be encountered and, as long as that temperature is known, the result can be corrected using the empirically determined Q_{10} relationship.

Incubation time

The rate of extracellular FP slows slightly over time during a Bulk FDA incubation. This could be caused by reduction in the cell membrane chemical gradients for FDA and fluorescein as FDA is converted to fluorescein. Depletion of extracellular FDA during the incubation will weaken the chemical gradient for FDA influx, while the chemical gradient of fluorescein efflux will decrease as extracellular fluorescein concentration climbs. Also, the rate of extracellular FP is expected to decrease after the cells being assayed have completed their phase of descending intracellular fluorescein concentration. The gradual slowing of extracellular FP during an incubation suggests that natural assemblages may have largely completed this descending phase after 1-hour. A 1-hour incubation improves signal/noise ratios and produces more readily detected fluorescent signals relative to shorter incubation times, and, for these reasons, has been

selected as the optimal incubation time. Varying incubation times close to 1 hour can be corrected for assuming a linear production of FP over time.

FDA concentration

One drawback of using FDA is that it has limited solubility in aqueous solvents (10µM) (Breeuwer et al., 1995). The increase in FP that is observed at FDA concentrations greater than 10µM is likely due to re-dissolution of precipitated FDA during the incubation period. Based on the maximum solubility of FDA, when FDA is added to Reagent A at 25µM, for example, approximately 10µM of the FDA is dissolved and 15µM is precipitated. As the enzymatic reaction proceeds, some of the dissolved FDA is converted to fluorescein, thus reducing the dissolved FDA concentration below 10µM. As much as 30% of the FDA that is added to a Bulk FDA incubation is converted to fluorescein after 1 hour under typical conditions. This depletion could allow some of the precipitated FDA to re-dissolve and become substrate for esterase activity, essentially serving as a source of FDA to replenish the dissolved substrate, resulting in more FP at the end of the incubation. Adding FDA at concentrations exceeding 10µM can be seen as an improvement to the assay because the substrate is in excess and a higher fluorescent signal is achieved with the same biomass loaded. However, abiotic FP increases more than biological FP at elevated FDA concentrations, resulting in a decreasing in signal/noise ratio. Also, the re-dissolution of precipitated FDA is likely a variable process, depending on the incubation temperature, amount of agitation to the samples during the incubation and possibly other factors. If the amount of precipitated FDA that becomes re-dissolved is variable between experiments, the relationship between the viable biomass loaded into the reaction vessel and FP will become more variable as well. In an effort to eliminate FDA concentration as a source of variability, it is recommended that FDA be added at 10µM.

Algal health in incubation buffer

Results from PAM measurements of cultures re-suspended in various incubation buffers suggest that 500mM Sorbitol + 20mM MES-KOH pH 6.5 likely causes some osmotic stress to freshwater organisms, and is better suited osmotically to accommodate

marine organisms. The freshwater alga, *Haematococcous*, showed reduced Fv/Fm values indicative of stress, yet remained intact, viable (Fv/Fm > 0.2) and capable of producing fluorescein at close to the expected rate, suggesting that this physiological stress caused by the incubation buffer may not cause changes in cellular EA. It is encouraging that the marine organisms showed little or no reduction in Fv/Fm. *Tetraselmis* sp. remained swimming in Reagent A, indicating that no major physiological change occurred during the incubation.

Effects of nutrients and light on cellular esterase activity

The growth experiment, using a batch culture of *Tetraselmis* sp., shows that during the period of exponential growth, the FP/cell ratio is relatively constant, making the Bulk FDA technique an excellent predictor of cellular growth rate. However, after this initial phase, the FP/cell decreases, which leads to an underestimate of the growth rate by Bulk FDA relative to that calculated from numeric counts (flow cytometry). Possible reasons for the decrease in FP/μm^3 include self shading by the culture as it became more dense, resulting in less light exposure, or nutrient depletion. This study showed that the FP/10-50μm cell increased when Moss Landing Harbor water was enriched with nutrients and placed in an illuminated incubator relative to a non-enriched, dark control. Brookes et al. (2000) found that EA/cell, defined as the rate of intracellular fluorescein accumulation measured by flow cytometry, was positively correlated with the light intensity used to culture cells. The same study also showed that EA/cell of nutrient limited cultures increased dramatically 1 day after the addition of the limiting nutrient (nitrogen or phosphorous). The quantity of ATP/cell has also been shown to decrease in nutrient limited algal cultures and increase 1 day following nutrient addition (Holm-Hansen 1970); the same study showed that light stimulated increases in cellular ATP. The finding that FP/cell correlates with the light and nutrient status of the cells is useful for making comparisons between FP from algal cultures and from natural samples. Cells in most natural environments are likely to be both nutrient limited (Howarth 1988) and light limited (Sunda et al. 1997), therefore, the FP/cell ratios observed in nutrient depleted, self-shaded cultures are likely more relevant for predicting the rate of FP/cell from natural samples.

Fluorescein production and ATP per biovolume as a function of cell size

The relationship between biovolume and FP was investigated by measuring the FP/cell from many different algal cultures spanning a wide size range, from *Synechococcus* (2.56μm^3/cell) to *Coscinodiscus* (582,000μm^3/cell). This study revealed a general trend of decreasing FP/μm^3 as cell size increased, ranging from 0.12 fg/hour.μm^3 by *Coscinodiscus* to 7.8 fg/hour.μm^3 by *Chlorella* (62 μm^3/cell), which results in a positive y-intercept on the plot of cell volume and FP (Figure 41). ATP was measured in tandem with Bulk FDA, and, interestingly, showed the same trend of decreasing ATP/μm^3 as cell size increased (Figure 42). This trend could be due in part to the fact that large sized algal cells have more vacuolar space than small cells. Vacuoles in algal cells are the sites of protein degradation and turnover, as well as providing cellular structure and immune function (Becker 2007). This vacuolar space contributes to cell volume but not to cellular carbon (Strathman 1967), and is likely to be less densely packed with esterase and ATP than in other areas of the cell (Verity 1992). It appears that smaller cells are more efficient at packing enzymes and ATP in a small intracellular space, while larger cells have more open intracellular space and less enzyme and ATP per unit cell volume, resulting in the trend of decreasing FP/μm^3 and ATP/μm^3 with increasing cell size observed in this study. It is also true that FP from large cells could be limited by FDA influx. Small cells have a higher surface area/volume ratio than large cells; FDA that enters cells will be exposed to the total pool of cytoplasmic esterases more rapidly in small cells than in large cells, which could result in underestimation of true cellular EA from large cells by the Bulk FDA technique.

Fluorescein production as a predictor of cell concentration

Regulatory standards for treated water, such as drinking water or ballast water, are defined as numeric concentration of viable cells in a defined size range. Ballast water discharge standards, for example, state that water to be discharged in a non-native port contain no more than 10 viable cells/mL for organisms 10-50µm (IMO G8, 2008). The relationship between FP and cell volume established by this study allow one to predict

the expected FP per cell rate for any sized cell. One can use this predicted FP/cell rate to convert a Bulk FDA assay result to a prediction of living cell concentration after assuming a single mean cell size for the sample. For example, for a size fractionated 10-50µm sample, one could assume that all living cells present in the sample are 10µm ESD (or, rather, that the mean cell size is 10µm ESD) and divide the FP result by the FP/cell rate of a 10µm cell to calculate the predicted cell concentration (cells/L). One could then follow the same logic under the assumption that all cells in the size fractionated sample were 50µm. This approach provides a generous and conservative estimate of concentration of living cells, which is useful for the testing of regulated water sources such as ballast water.

The validity of this approach can be investigated by comparing the FP/cell rates of cultured algal cells of known cell size with FP/cell rates of size fractionated (10-50µm) natural samples collected coastally around Monterey Bay. According to the regression between cell volume and FP (Figure 41), a 10 µm cell has a predicted FP rate of 0.72 pg/cell.hr, while a 50 µm cell has a predicted FP rate of 35 pg/cell.hr. Each coastal Monterey Bay sample was measured by both Bulk FDA (collected on 10µm filters) and by flow cytometry for numeric concentration of live cells 10-50µm, enabling the calculation of per 10-50µm cell FP rates. These rates were highly variable (coefficient of variation = 177%), ranging from 0.1-17 pg/cell.hr, with a mean of 2.6 pg/cell.hr and median of 1.2 pg/cell.hr. All cells contributing to these counts are between 10-50µm, but the actual size of cells within this range is not known. Variation in the dominant cell size among sample sites and days likely contributed towards the high variance observed in FP/cell rates. Conversion of the coastal Monterey Bay FP/cell rates to an estimate of mean cell size yields a range of 5-39 µm (ESD) with a mean of 18 µm and a median of 13 µm. Estimates from both the mean and median fall within the 10-50 µm range, which is encouraging, as only cells between 10-50 µm are present in these size fractionated samples. Both of these estimates are towards the lower end of the 10-50 µm range, which is in agreement with size distribution trends expected based on decades of research which shows that both the abundance and biomass of organisms in aquatic environments is inversely related to cell size (Cavender-Bares et al., 2001). This suggests that the

relationship between cell volume and FP established using algal cultures is useful in predicting the live cell concentration of natural samples assayed by Bulk FDA.

Fluorescein Production and ATP

The relationship between FP and ATP concentration was investigated in detail in this study using algal cultures, surface to deep water Monterey Bay samples and coastal Monterey Bay samples (Figure 39). In all cases, FP and ATP are positively correlated, which confirms that the Bulk FDA technique measures viable biomass in the same manner as ATP. However, the relationship between these indicators is not strictly proportional. Log transformed regression of these two parameters (ATP on x-axis, FP on y-axis) using all coastal and offshore/deep Monterey Bay samples reveals that ATP varies over a greater range than FP, resulting in a slope of 0.67 (Figure 40a). The same is true of the algal culture data (slope of log-log regression=0.85). It is possible that a cause of this discrepancy is that too small a blank value is being used for Bulk FDA, resulting in higher than expected FP values in low biomass samples. An empirical "blank" is subtracted from both the ATP and Bulk FDA values to correct for any non-biological signal. For all ATP vs. FP comparisons, this blank consisted of FSW filtered and processed in the same way as samples. It was observed that the discrepancy between the ranges spanned by FP and ATP could be corrected if a Bulk FDA blank value 3-times larger than the empirical blank was substituted; both viable biomass indicators then vary over approximately the same range, resulting in a slope approaching 1 on the log (FP) vs. log(ATP) regression (actual slope value =0.92) (Figure 40b). However, no justification for using a larger Bulk FDA blank has been found. Samples killed with high concentrations of bleach and processed for Bulk FDA show FP values similar to the FSW blank, suggesting that non-biological particles are not the cause of the high FP/ATP ratios observed in low biomass samples.

It is also possible that the discrepancy between the range spanned by ATP and FP is not caused by low biomass samples, but rather samples in which high levels of biomass are loaded. It has been shown that FP per unit cell volume decreases as cell volume increases, possibly due to inefficiency of exposing all cellular esterases to FDA in large cells. In samples in which large amounts of biomass are collected on a filter, FDA may

not come in contact with cells hidden under a layer of material, resulting in lower FP than if all cells had been exposed. The boiling extraction of ATP may be more efficient at capturing all ATP in these high biomass samples than the Bulk FDA technique is at exposing all esterases to FDA, resulting in ATP spanning a greater range than FP.

Figure 38: Fluorescein production vs. ATP comparisons from 3 sources plotted together: water column profiles offshore in Monterey Bay, coastal Monterey Bay samples, and freshwater and marine algal cultures

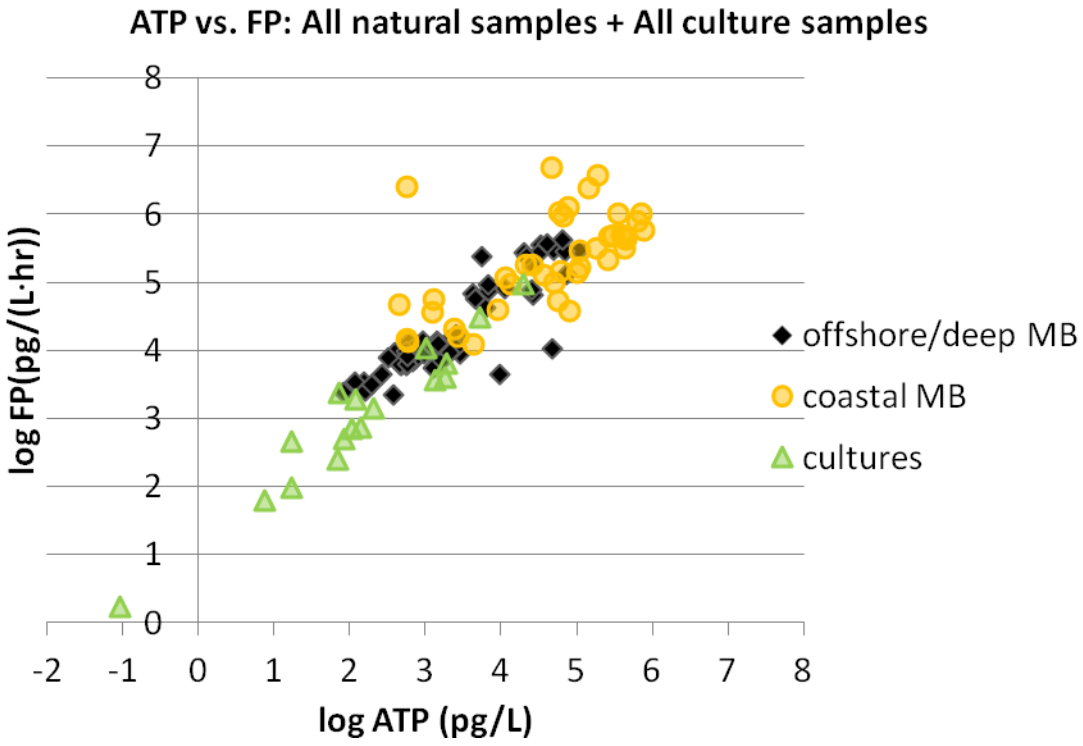
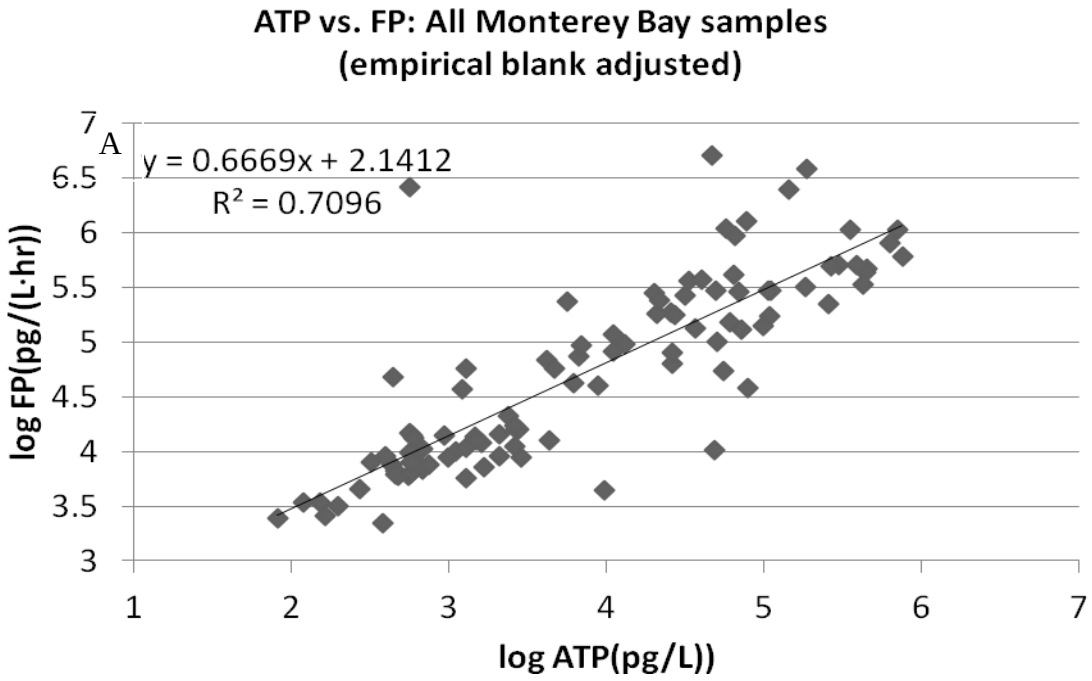
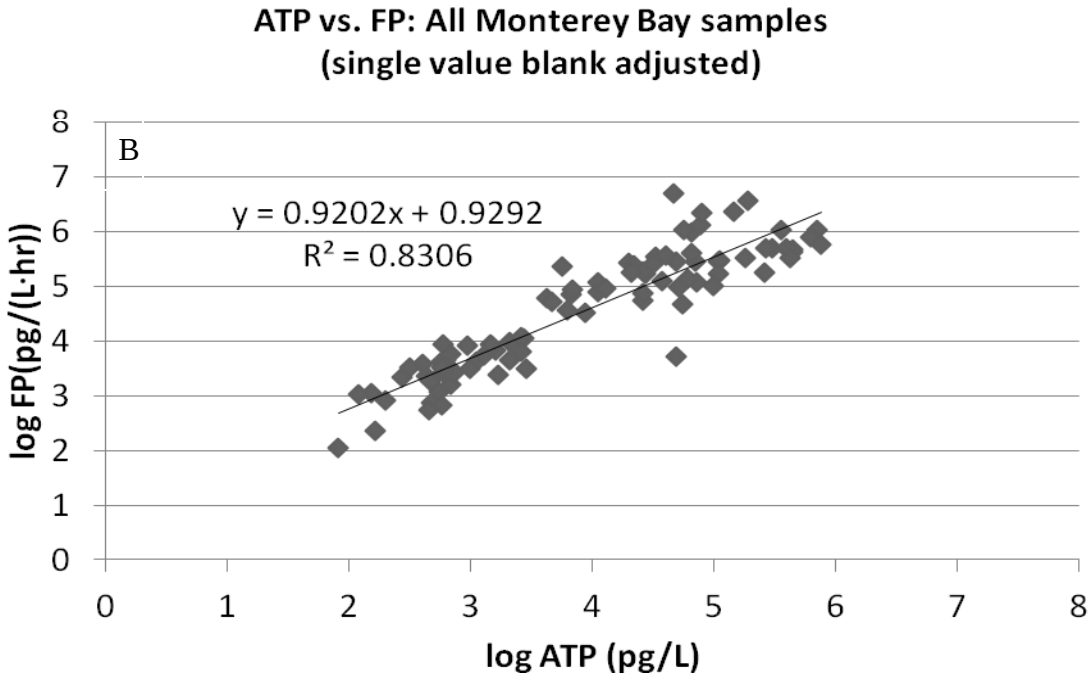


Figure 39: a) The relationship between FP and ATP from Monterey Bay samples when the FP value is corrected by an empirical, cell-free blank b) The same relationship using FP values corrected by substituting a single value blank of 3x higher fluorescence than the empirical blank





CONCLUSION

Optimization of the Bulk FDA technique has resulted in an assay that is greatly improved in terms of sensitivity, precision, accuracy and ease of use. Moreover, knowledge obtained during the optimization process give the user the option of modifying aspects of the assay, such as pH, temperature, incubation time or volume, to suit their particular needs. The optimized assay has adequate sensitivity to be used not only in natural environments, but also to evaluate sterilized water, such as treated ballast water, which contains little or no living organisms**.** The relationships established by this thesis allow one to convert the bulk measure of living biomass generated by the Bulk FDA technique into a numeric estimate of living cell concentration. The Bulk FDA assay is reliable, convenient, and can be carried out using portable instrumentation over a wide range in ambient temperatures by those with very little technical training. This combination of features makes the Bulk FDA technique a unique tool and will promote its usefulness in a wide array of academic and regulatory applications.

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